Aos meus pais

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Greenhouse gas reduction through innovative nitrogen removal from wastewater

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Reductie van broeikasgassen door innovatieve stikstofverwijdering uit afvalwater.

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Photo taken on the clear waters of the Ons Island in Galicia (Spain) by myself and modified with the help of my brother Jose C. Castro Barros.

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List of abbreviations

Abbreviation	Description
AC	Analysis Control
AMO	ammonia monooxygenase enzyme
amoA	ammonia monooxygenase gene
anammox	ANaerobic AMMonium OXidation
AOA	Ammonium Oxidizing Archaea
AOB	Ammonium Oxidizing Bacteria
AOR	Ammonium Oxidation Rate
BNR	Biological Nitrogen Removal
CANON	Completelyy Autotrophic Nitrogen removal Over Nitrite
CoA	Coenzyme A
COD	Chemical Oxygen Demand
damo	denitrifying anaerobic methane oxidation
damoA	denitrifying anaerobic methane oxidizing Archaea
damoB	denitrifying anaerobic methane oxidizing Bacteria
DNRA	Dissimilatory Nitrate Reduction to Ammonium
DO	Dissolved Oxygen
EPA	Environmental Protection Agency
FIA	Flow Injection Analysis
FISH	Fluorescence In Situ Hybridization
FT	Flow Transmitter
GC	Gas Chromatography
HAO	hydroxylamine oxidoreductase enzyme
HPLC	High Performance Liquid Chromatography
HRAS	High Rate Activated Sludge
HRT	Hydraulic Retention Time
IPCC	Intergovernmental Panel on Climate Change
MeOH	Methanol
MLSS	Mixed Liquor Suspended Solids

MLVSS	Mixed Liquor Volatile Suspended Solids
MOB	aerobic Methane Oxidizing Bacteria
N	Nitrogen
NAP	periplasmic nitrate reductase enzyme
NAR	membrane-bound nitrate reductase enzyme
nir	nitrite reductase gene
nirK/nirS	nitrite reductase enzymes
NOB	Nitrite Oxidizing Bacteria
NOR	nitric oxide reductase enzyme
NOS	nitrous oxide reductase enzyme
NXR	nitrite oxidoreductase enzyme
PAO	Phosphate Accumulating Organism
PHB	Poly-β-HydroxyButyrate
PLC	Programmable Logic Controller
PNA	Partial Nitritation-Anammox
SBR	Sequencing Batch Reactor
SBR	Solid/Sludge Retention Time
SCADA	Supervisory Control And Data Acquisition
SHARON	Single reactor system for High activity Ammonium Removal Over Nitrite
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solids
WWTP	WasteWater Treatment Plant

List of symbols

Symbol	Description	Unit
a_1	Parameter obtained from data fitting	g N m^{-3}
a_2	Pre-exponential factor - parameter obtained from data fitting	g N m^{-3}
a_3	Exponential factor - parameter obtained from data fitting	g N m^{-3}
a_4	Pre-exponential factor - parameter obtained from data fitting	g N m^{-3}
a_5	Exponential factor - parameter obtained from data fitting	g N m^{-3}
b_X	Specific biomass decay rate of X	d^{-1}
$C_{G,NO2}$	Gas N_2O concentration	g N m^{-3}
$C_{L,N2O}$	Liquid N_2O concentration	g N m^{-3}
C_y	Concentration of compound y	g y m^{-3}
D_L	Liquid phase dilution rate	min^{-1}
EF_y	Emission factor of compound y	% (w/w)
f_I	Inert content in biomass	g COD (g COD)$^{-1}$
H	Height	M
i_{NSS}	Nitrogen content in organic substrate	g N (g COD)$^{-1}$
i_{NXB}	Nitrogen content in active biomass	g N (g COD)$^{-1}$
i_{NXI}	Nitrogen content in the inert fraction	g N (g COD)$^{-1}$
K_{CH4}^X	Methane half saturation constant of X	g COD m^{-3}
$K_{i\text{-}NO2}^X$	Nitrite inhibition constant of X	g N m^{-3}
$K_{i\text{-}O2}^X$	Oxygen inhibition constant of X	g O_2 m^{-3}
k_La_y	Gas-liquid mass transfer coefficient of compound y	d^{-1}
K_{NH4}^X	Ammonoum half saturation constant of X	g N m^{-3}
K_{NO2}^X	Nitrite half saturation constant of X	g N m^{-3}

K_{NO3}^{X}	Nitrate half saturation constant of X	g N m^{-3}
Symbol	**Description**	**Unit**
K_{O2}^{X}	Oxygen half saturation constant of X	g O_2 m^{-3}
μ_{max}^{X}	Maximum growth rate of X	d^{-1}
M_y	Mass of compound y	g y (g VSS)$^{-1}$ h^{-1}
Q_G	Gas flow rate	m^3 h^{-1}
Q_L	Liquid flow rate	m^3 h^{-1}
$R_{V,N2O}$	N_2O formation rate	g N m^{-3} min^{-1}
r_y	Specific consumption or production rate of compound y	g y (g VSS)$^{-1}$ h^{-1}
S_y	Concentration of compound y	g y m^{-3}
TR_y	Transfer rate for compound y	kg y m^{-3} s^{-1}
V_G	Headspace volume	m^3
V_L	Liquid volume	m^3
V_R	Reactor volume	m^3
X_X	Biomass concentration of X	g COD m^{-3}
Y_X	Biomass yield of X	g COD (g N)$^{-1}$ or (g COD)$^{-1}$

Superscripts

HighSS	Steady state of the high aeration period
HighT	Transient to the high aeration period
Inf	Influent
Low	Low aeration period

Subscripts

L0	Start of the low aeration period
Lf	End of the low aeration period
t0	Start of the interval
tn	End of the interval

Summary

Greenhouse gas emissions due to human activities are higher than ever, having an important contribution to the fast climate change that the world is experiencing nowadays (IPCC 2014b).

During wastewater handling greenhouse gases are emitted. Carbon dioxide (CO_2) is produced mainly during the combustion of fossil fuels to generate energy and electricity for the wastewater treatment plant; nitrous oxide (N_2O) is emitted during biological nitrogen removal systems; and methane (CH_4) can be released after its formation in the sewage system or in anaerobic digestion systems. Taking CO_2 as reference gas, N_2O has a global warming potential of 298-CO_2 equivalents over 100 year horizon, while CH_4 accounts for 34-CO_2 equivalents (IPCC 2013). Therefore, even small emissions of N_2O and CH_4 may contribute significantly to the carbon footprint of the wastewater treatment plant.

The objective of this thesis is to minimize the emissions of the greenhouse gases CO_2, N_2O and CH_4 during wastewater treatment by optimizing the operation of processes for biological nitrogen removal and by applying innovative nitrogen removal technologies. Both mainstream (low strength ammonium, high organic carbon content, cold) and sidestream (ammonium-rich, warm) wastewaters are studied.

Chapter 1 gives a general introduction on biological techniques for nitrogen removal from wastewater and on greenhouse gas emissions from wastewater treatment plants, describing the known sources of CO_2, N_2O and CH_4, as well as the pathways for N_2O production. Process options to reduce greenhouse gas emissions from wastewater treatment are pointed out. The anaerobic ammonium oxidation (anammox) reaction, comprising the simultaneous conversion of ammonium and nitrite to nitrogen gas, is put forward as a more sustainable option for biological nitrogen removal from wastewater, compared to conventional nitrification-denitrification over nitrate, since it takes up to 63% less aeration energy, thus producing less CO_2.

While the application of anammox for sidestream treatment has become relatively widespread, its implementation in the mainstream of the

wastewater treatment plant is very challenging. One of the main bottlenecks is the suppression of the nitrite oxidizers (oxidation of nitrite to nitrate), which is troublesome under mainstream conditions (Lotti et al. 2015). It was recently discovered that anammox bacteria are not only capable of the conventional (autotrophic) anammox reaction, but can also reduce nitrate via nitrite to ammonium coupled with the oxidation of organic compounds (Guven et al. 2005; Kartal et al. 2007a). The potential application of this organotrophic nitrate reduction capacity of anammox bacteria for the treatment of **mainstream wastewater containing organic carbon** was investigated in **Chapter 2**. Batch tests with anammox biomass as such and together with heterotrophic bacteria were performed under different feeding conditions; a sequencing batch reactor was operated with semi-continuous acetate and nitrate feeding. The Gibbs free energy from different reactions was calculated for better understanding of the competition for nitrate and organic carbon between autotrophic anammox bacteria and heterotrophic organisms. The capacity of anammox bacteria to reduce nitrate to nitrite (and not further to ammonium), together with the conventional anammox conversion, was confirmed. Factors promoting this combined conversion by anammox bacteria (reduction of nitrate to nitrite + anammox reaction) over heterotrophic transformations were identified, such as the presence of ammonium in the medium, limiting organic carbon conditions and sufficient amount of inorganic carbon present. Finally, a process scheme including the combined nitrate reduction to nitrite by anammox bacteria and the typical anammox reaction was proposed for mainstream treatment of municipal wastewater, which would result in about 50% less energy consumption compared to conventional nitrogen removal systems based on nitrification-denitrification over nitrate, and thus less $\mathbf{CO_2}$ emissions.

When establishing nitrite-based technologies for nitrogen removal from municipal wastewater, such as anammox-based systems, nitrite can accumulate, promoting $\mathbf{N_2O}$ emissions (Colliver & Stephenson 2000). Also, occasional accumulation of nitrite may take place during conventional nitrogen removal systems from mainstream wastewater depending on the operating conditions and loading rates of the plant. The effect of nitrite shocks

and nitrite accumulation on the production of N_2O and nitric oxide (NO) in a nitrifying lab-scale reactor treating a synthetic **low strength ammonium wastewater** was assessed in **Chapter 3**. Online measurements of N_2O and NO in the off-gas of the reactor were carried out to study the impact of different nitrite dosing strategies on the emission dynamics. The results showed the enhancement of N_2O and NO emissions under nitrite accumulation, with a stronger effect on NO emissions at high nitrite concentrations. The main responsible pathway for the emissions was the autotrophic nitrite reduction by aerobic ammonium oxidizers, which implies that NO cannot be neglected as intermediate during the formation of N_2O during this pathway. Also, high nitrite concentrations inhibited the nitrifying activity of the non-adapted populations to nitrite. In general, the results contribute to understand the formation of N_2O and NO in case of momentary nitrite accumulation and their potential reduction during municipal wastewater treatment.

During the treatment of wastewater with **high ammonium concentrations**, such as reject water from anaerobic digestion, **N_2O** emissions are potentially very significant, for instance, because of high nitrite accumulation. In this regard, **Chapter 4** deals with N_2O emissions during the treatment of ammonium-rich wastewater through partial nitritation-anammox in a single reactor. An online N_2O monitoring campaign was carried out at a full-scale partial nitritation-anammox granular sludge reactor, assessing the effect of the aeration regime on the N_2O emissions during the normal reactor operation and during a dedicated experiment. From the gas-phase N_2O measurements, the N_2O formation rate and the N_2O liquid concentration profiles were calculated. The results showed that aeration is an important influencing parameter on the N_2O emission dynamics. The N_2O emission was higher during intense aeration, not only due to enhanced stripping, but also because of a larger N_2O formation rate. Anoxic periods led to N_2O consumption, likely by heterotrophic denitrification. Since the highest N_2O formation rates were obtained during the transient from anoxic (or low aerated) to aerated periods, it is recommended to keep shifts in the aeration pattern smooth in order to minimize N_2O emissions. Overall, it was shown

that an adequate aeration control strategy may reduce N_2O emissions from one-stage partial nitritation-anammox systems.

Apart from a high ammonium concentration, **reject water** contains also dissolved **CH_4**. In **Chapter 5,** the integration of CH_4 removal from ammonium-rich wastewater in aerobic granular sludge anammox-based reactors was assessed. A model was set up to study the substrate competition among denitrifying anaerobic methane oxidizing bacteria and archaea, aerobic methane oxidizers, and anammox bacteria, besides heterotrophs, aerobic ammonium and nitrite oxidizers. Simulations were carried out under different ammonium and oxygen concentrations. The results showed that simultaneous CH_4 and nitrogen removal is feasible under oxygen limited conditions. For the different ammonium concentrations evaluated, the maximum CH_4 removal was always achieved under the same dissolved oxygen conditions as for maximum nitrogen removal. CH_4 removal was mainly realized by aerobic methanotrophs. Mitigating stripping strategies were investigated to reduce potential CH_4 stripping in aerated systems. It was found that locating the aeration supply deeper in the reactor can significantly reduce CH_4 stripping, since the gas solubility is increased in this way . These findings may lead to the potential integration of CH_4 removal in aerobic anammox-based granular sludge reactors in practice.

Chapter 6 summarizes the main conclusions from this thesis**.** The exploitation of the nitrate reduction capacity of anammox bacteria could facilitate the implementation of the anammox reaction during mainstream wastewater treatment, thus reducing the energy consumption of the plant and the **CO_2** emissions. **N_2O** emission could be reduced by adjusting operational parameters, namely by minimizing the nitrite accumulation and through an appropriate aeration control strategy. Also, **CH_4** removal from reject water could be integrated during aerobic anammox-based reactors. Overall, this thesis contributes to the reduction of greenhouse gas emissions during nitrogen removal from wastewater by optimizing process operation and contributing to the further development and application of innovative anammox-based technologies by integrating novel conversions.

Samenvatting

De emissie van broeikasgassen door menselijke activiteit ligt hoger dan ooit, en draagt in belangrijke mate bij aan de snelle klimaatverandering die de wereld vandaag ervaart (IPCC 2014b).

Bij de behandeling van afvalwater worden broeikasgassen uitgestoten. Koolstofdioxide (CO_2) wordt vooral geproduceerd bij de verbranding van fossiele brandstoffen om energie en elektriciteit op te wekken voor de waterzuiveringsinstallatie; lachgas (distikstofoxide,N_2O) wordt uitgestoten tijdens biologische stikstofverwijderingsprocessen; en methaan (CH_4) kan vrijkomen na vorming in de riolen of tijdens anaerobe vergistingsprocessen. In vergelijking met CO_2, heeft N_2O een *global warming potential* van 298 CO_2-equivalent over een periode van 100 jaar, terwijl CH_4 voor 34 CO_2-equivalent telt (IPCC 2013). Daarom kunnen zelfs kleine emissies van N_2O en CH_4 significant bijdragen tot de koolstofvoetafdruk van de waterzuiveringsinstallatie.

De doelstelling van deze scriptie is het verkleinen van de emissies van de broeikasgassen CO_2, N_2O en CH_4 bij waterzuivering door optimalisatie van processen voor biologische stikstofverwijdering te optimaliseren en door het toepassen van innovatieve stikstofverwijderingstechnologieën. Zowel afvalwaters in de hoofdstroom (laag aan ammonium, rijk aan organische koolstof, koud) als in de zijstroom (rijk aan ammonium, warm) van waterzuiveringsinstallatiesworden bestudeerd.

Hoofdstuk 1 geeft een algemene inleiding met betrekking tot biologische technieken voor de stikstofverwijdering uit afvalwater en broeikasgasemissies uit waterzuiveringsinstallaties. Hierbij worden zowel de gekende bronnen van CO_2, N_2O en CH_4 beschreven, als de manieren waarop N_2O wordt geproduceerd. Procesopties om de broeikasgasemissies bij waterzuivering te reduceren worden aangetoond. De anaerobe ammoniumoxidatie (anammox) reactie, die een simultane conversie van ammonium en nitriet naar stikstofgas omvat, wordt vooropgesteld als een meer duurzame optie voor biologische stikstofverwijdering uit afvalwater, in vergelijking met conventionele nitrificatie-denitrificatie via nitraat, aangezien

deze tot 63% minder beluchtingsenergie vereist, en zo minder CO_2 produceert.

Terwijl de toepassing van anammox voor de behandeling van de nevenstroom relatief wijdverspreid is, is de implementatie ervan in de hoofdstroom van de waterzuiveringsinstallatie een echte uitdaging. Een van de voornaamste problemen is de onderdrukking van de nitrietoxideerders (oxidatie van nitriet tot nitraat), wat moeilijk is onder de omstandigheden in de hoofdstroom (Lotti et al. 2015). Recent werd ontdekt dat anammoxbacteriën niet alleen in staat zijn de conventionele (autotrofe) anammox-reactie uit te voeren, maar ook nitraat via nitriet tot ammonium kunnen reduceren, gekoppeld aan de oxidatie van organische koolstof (Guven et al. 2005; Kartal et al. 2007a). De potentiële toepassing van deze organotrofe nitraatreductiecapaciteit van anammoxbacteriën voor de behandeling van **hoofdstroomafvalwater dat organische koolstof bevat**, werd onderzocht in **hoofdstuk 2**. Batchtests met anammoxbiomassa op zich en samen met heterotrofe bacteriën werden uitgevoerd onder verschillende voedingscondities; een sequencing batch reactor werd bedreven met semi-continue toevoer van acetaat en nitraat. De Gibbs vrije energie bij verschillende reacties werd berekend voor een beter begrip van de concurrentie voor nitraat en organische koolstof tussen autotrofe anammoxbacteriën en heterotrofe organismen. De capaciteit van anammoxbacteriën om nitraat te reduceren tot nitriet (en niet verder tot ammonium), naast de conventionele anammox-conversie, werd bevestigd. De factoren die deze gecombineerde omzetting (reductie van nitraat tot nitriet en de anammox reactie) bevorderen ten opzichte van heterotrofe omzetting werden geïdentificeerd, zoals de aanwezigheid van ammonium in het medium, limiterende omstandigheden voor organische koolstof en de voldoende aanwezigheid van anorganische koolstof. Tenslotte werd een processchema voorgesteld voor de behandeling van huishoudelijk afvalwater in de hoofdstroom, met inbegrip van de gecombineerde nitraatreductie tot nitriet door anammoxbacteriën en de typische anammoxreactie. Dit processchemazou resulteren in ongeveer 50% minder energieverbruik in

vergelijking met conventionele stikstofverwijderingssystemen gebaseerd op nitrificatie-denitrificatie via nitraat, en aldus minder **CO_2** emissies.

Bij het gebruik van nitrietgebaseerde technologieën voor de stikstofverwijdering uit huishoudelijk afvalwater, zoals anammox-gebaseerde systemen, kan nitriet accumuleren, wat de uitstoot van **N_2O** bevordert (Colliver & Stephenson 2000). Occasionele accumulatie van nitriet kan ook plaatsvinden in conventionele stikstofverwijderingsprocessen voor de behandeling van huishoudelijk afvalwater in de hoofdstroom, afhankelijk van de procesomstandigheden en de belasting van de installatie. Het effect van nitrietschokken en nitrietaccumulatie op de productie van N_2O en stikstofoxide (NO) in een nitrificerende laboratoriumschaalreactor voor de behandeling van een synthetisch afvalwater met laag ammoniumgehalte, werd geëvalueerd in **Hoofdstuk 3**. Via online metingen van N_2O en NO in de uitgaande gasstroom van de reactor werd de impact van verschillende nitrietdoseringsstrategieën op de emissiedynamiek bestudeerd. De resultaten toonden de verhoging van N_2O en NO-emissies bij nitrietaccumulatie aan, met een sterker effect op NO-emissies bij hoge nitrietconcentraties. De belangrijkste veroorzaker van de emissies was de autotrofe nitrietreductie door aerobe ammoniumoxideerders, wat inhoudt dat NO niet kan worden verwaarloosd as intermediair bij de vorming van N_2O langs deze weg. Bovendien verhinderden hoge nitrietconcentraties de nitrificatie door de populaties die niet zijn aangepast aan nitriet. Over het algemeen dragen de resultaten bij aan het begrip van de vorming van N_2O en NO in het geval van tijdelijke nitrietaccumulatie en hun potentiële vermindering bij de behandeling van huishoudelijk afvalwater.

Tijdens de behandeling van afvalwater met **hoge ammoniumconcentraties**, zoals rejectiewater afkomstige van anaerobe vergisting, zijn **N_2O**-emissies potentieel erg significant, bijvoorbeeld omwille van de hoge accumulatie van nitriet. In deze optiek handelt **Hoofdstuk 4** over N_2O-emissies bij de behandeling van ammoniumrijkafvalwater door partiële nitritatie-anammox in een enkele rector. Een online N_2O meetcampagne werd uitgevoerd in een volleschaal-korrelslibreactor voor nitritatie-anammox, waarbij het effect van

het beluchtingsregime op de N_2O-emissies werd nagegaan bij een normale werking van de reactor en tijdens een specifiek experiment. Op basis van de N_2O-metingen in de gasfase, werden de N_2O-vormingssnelheid en de N_2O concentratieprofielen in de vloeistof berekend. De resultaten toonden aan dat beluchting een belangrijke invloed heeft op de N_2O-emissiedynamica. De N_2O-emissie was hoger tijdens intense beluchting, niet alleen door verhoogde stripping, maar ook door een hogere N_2O-vormingssnelheid. Zuurstofloze periodes leidden tot N_2O-consumptie, vermoedelijk door heterotrofe denitrificatie. Aangezien de hoogste N_2O-vormingssnelheden werden verkregen tijdens de overgangsfase tussen zuurstofloze (of weinig beluchte) en beluchte periodes, is het aanbevolen om veranderingen in het beluchtingspatroon gelijkmatig te houden, om zo de N_2O-emissies te reduceren. In het algemeen werd aangetoond dat een geschikte beluchtingsstrategie de N_2O-emissies van een eentraps partieel nitritatie-anammox systeem kan verminderen.

Naast een hoge ammoniumconcentratie, bevat **rejectiewater** afkomstig van anaerobe vergisting ook opgeloste **CH_4**. In **Hoofdstuk 5** wordt de integratie van CH_4-verwijdering uit ammoniumrijk afvalwater in aerobe anammox-gebaseerde korrelslibreactoren toegepast. Een model werd opgesteld voor het bestuderen van de competitie voor substraat tussen denitrificerende anaerobe methaanoxiderende bacteriën en archaea, aerobe methaanoxideerders en anammoxbacteriën, naast heterotrofen en aerobe ammonium- en nitrietoxideerders. Hiertoe werder simulaties uitgevoerd bij verschillende ammonium- en zuurstofconcentraties. De resultaten toonden aan dat simultane CH_4- en stikstofverwijdering mogelijk is onder beperkende zuurstofcondities. Voor de verschillende concentraties ammonium die werden geëvalueerd, vielen de optimale zuurstofconditiesvoor maximale CH_4-verwijdering steeds samen met die voor maximale stikstofverwijdering. CH_4-verwijdering werd hoofdzakelijk gerealiseerd door aerobe methanotrofen. Er werd verder onderzocht hoe de mogelijke stripping van CH_4 in beluchte systemen kan worden tegengegaan. Hierbij werd vastgesteld dat CH_4-stripping significant kan worden verminderd door de beluchter diep in de reactor te plaatsen, aangezien op die manier de gasoplosbaarheid

verhoogd wordt. Deze bevindingen kunnen leiden tot de potentiële integratie van CH_4-verwijdering in aerobe anammox-gebaseerde korrelslibreactoren in de praktijk.

Hoofdstuk 6 vat de belangrijkste conclusies van deze scriptie samen. De exploitatie van de nitraatreductiecapaciteit van anammoxbacteriën zou de implementatie van de anammox-reactie voor de behandeling van huishoudelijk afvalwater in de hoofdstroom kunnen faciliteren, en aldus bijdragen tot de vermindering van het energieverbruik en de $\mathbf{CO_2}$-emissies van de installatie. $\mathbf{N_2O}$-emissies kunnen gereduceerd worden door het aanpassen van bedrijfsparameters, met name door het tegengaan van nitrietaccumulatie en door een geschikte regelstrategie voor de beluchting. Daarnaast zou de verwijdering van $\mathbf{CH_4}$ uit afvalwater geïntegreerd kunnen worden bij aerobe anammoxgebaseerde reactoren. In het algemeendraagt deze scriptie bij tot de reductie van broeikasgasemissies tijdens de stikstofverwijdering uit afvalwater door het optimaliseren van de procesvoering en tot de verdere ontwikkeling en toepassing van innovatieve anammoxgebaseerde technologieën door integratie van nieuwe bioconversies.

1

Introduction

1.1 Wastewater treatment sustainability

Current wastewater treatment technologies are able to produce clean effluents that meet legislation requirements. However, are they also sustainable? According to the definition of sustainability of the US Environmental Protection Agency, a sustainable system or process needs to keep its *own viability by using techniques that allow humans and nature to exist in productive harmony, to support present and future generations*.

Sustainability involves social, environmental and economic aspects. Energy is one of the most important factors concerning the sustainability of wastewater treatment plants due to its economic impact. Many studies are focusing in achieving energy self-sufficient or energy yielding wastewater treatment facilities (Garrido et al. 2013; Kartal et al. 2010; Meerburg et al. 2015; Siegrist et al. 2008). These studies discus and propose novel schemes that can save aeration energy, for instance by using novel nitrogen removal anammox-based processes (section 1.4) or high rate organic carbon removal technologies, and recover chemical energy contained in the wastewater by producing methane rich biogas.

Apart from energy recovery (methane rich biogas), water, nutrients and organics can be recovered from wastewater (Batstone et al. 2015; Kleerebezem et al. 2015; Verstraete et al. 2009). Water could be reused after polishing steps with membranes after the activated sludge treatment. The recovery of nutrients (nitrogen and phosphorus) highly depends on the market demand and price. Phosphorus recovery is applied in many plants by means of biological processes based on phosphate accumulating organisms (PAOs) or chemical precipitation, such as struvite precipitation. Nitrogen (as ammonia and/or nitrate) can be obtained through air stripping or also precipitation. Both phosphorus and nitrogen can be used as fertilizers. Regarding nitrogen, due to the high cost to recover it, so far it seems more beneficial to eliminate the ammonium in the wastewater. While the current trend is focusing on energy recovery from the main line and still removing nitrogen (Batstone et al. (2015), other approaches combine energy production with partial ammonia recovery (Verstraete & Vlaeminck 2011).

The production of organics from the organic matter of the wastewater is also gaining interest as an alternative to the production of methane biogas through anaerobic digestion (Kleerebezem et al. 2015).

Other important sustainability aspects related to human health and environmental impact are the discharge of heavy metals contained in the sludge, which can be incorporated into trophic chains and micropollutants or pharmaceutical compounds. Also, the emission of greenhouse gases to the atmosphere is of high concern and attracts a lot of attention due to the negative impact on climate change. In general, life cycle assessments (LCA) are useful methods to evaluate the different sustainability aspects from wastewater treatment, determining the relative importance comparing the different solution to get a sustainable process (Lorenzo-Toja et al. 2016; Schaubroeck et al. 2015).

1.2 General objective of this work

This thesis focuses on the minimization of the greenhouse gases carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) during urban wastewater treatment. More specifically, the potential greenhouse gas reduction during nitrogen removal from wastewater treatment is aimed at through optimized process operation and through the development and application of innovative based technologies, integrating novel conversions. Both mainstream (low strength ammonium, high organic carbon concentration, cold) and sidestream (ammonium-rich, warm) wastewaters are studied. The applied methodology concerns lab-scale experiments, a full-scale measurement campaign and mathematical modelling and simulation.

1.3 Why do we care about greenhouse gas emissions?

Climate change is a natural event, but in the past 60 years the Earth has experienced an abrupt climate alteration due to the increasing anthropogenic greenhouse gas emissions, which are higher than ever (IPCC 2014b). The main human activities that contribute to the rise of greenhouse gas emissions are

agriculture, transport and industry, including the waste and wastewater field (IPCC 2014a).

The direct consequence of this excessive greenhouse gas emissions is a rapid global warming. Since the 19th century, when the industrial period started, the average temperature in the atmosphere has increased with 0.85 °C and with 0.11 °C on the top layer of the ocean (IPCC 2013). The resulting changes in the natural ecosystems are significant. 30% of the total anthropogenic CO_2 emitted has been absorbed by the ocean, resulting in its acidification, and the extension of snow and ice is fastly decreasing. The ocean level increased more in the 20th century than during the previous 2700 years. This level increase was at least double than it would have been without anthropogenic contribution to the climate change (increase of 14 cm vs. -3 to 7 cm, respectively) (Kopp et al. 2016). Also the water cycle is altered noticeably, in terms of more abrupt changes between wet and dry regions and sessions (rainfalls and droughts), and unusual severe weather phenomena. This alteration in the prevailing ecosystems has social consequences as well, hindering the life in certain regions and triggering migration movements.

1.4 Nitrogen removal from wastewater treatment

Effective nitrogen removal from wastewater is required. High concentrations of ammonia, nitrate and nitrite in natural water bodies are toxic for living organisms (Camargo et al. 2005; Randall & Tsui 2002) and excess nutrients (nitrogen and phosphorus) promote eutrophication. The latter phenomenon is caused by an excessive algae growth, which impedes the penetration of sunlight producing the death of aquatic flora and therefore the depletion of oxygen due to the decomposition of the death plants. Biological methods for the removal of ammonium, the dominant nitrogen form in wastewater, have proven their effectiveness and are widely applied. The following sections give an overview of relevant nitrogen conversions in wastewater treatment and pay particular attention to the anammox technology and the bottlenecks for its application to mainstream wastewater treatment.

1.4.1 Nitrification-denitrification

Nitrogen (ammonium) is typically removed from wastewater by means of nitrification-denitrification over nitrate. Nitrification concerns the oxidation of ammonium to nitrite (nitritation) by ammonium oxidizing bacteria (AOB) or ammonium oxidizing archaea (AOA) (Konneke et al. 2005) (Eq. 1.1) and subsequently to nitrate (nitratation) by nitrite oxidizing bacteria (NOB) (Eq. 1.2) under aerobic conditions.

$$NH_4^+ + 1.5\ O_2 \rightarrow\ NO_2^- + H_2O + 2H^+ \quad [1.1]$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad [1.2]$$

Both ammonium and nitrite oxidizers are chemolithoautotrophic microorganisms, using inorganic carbon to grow. Moreover, it was recently discovered that some NOB (*Nitrospira*) are capable of performing full nitrification, i.e. to oxidize ammonium via nitrite to nitrate (Daims et al. 2015; van Kessel et al. 2015).

During denitrification, nitrate and/or nitrite are reduced to nitrogen gas (Eq. 1.3), usually by heterotrophic microorganisms under anoxic conditions. Heterotrophic bacteria use organic carbon compounds as electron donor, which need to be added to the wastewater (typically in the form of methanol) when not present in sufficient amount.

$$NO_3^- \rightarrow\ NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow\ N_2 \quad [1.3]$$

Much less common are some heterotrophic organisms that are able to perform nitrification and reduce nitrate and nitrite under aerobic conditions, hence coupling nitrification-denitrification (Hooijmans et al. 1990; Zhang et al. 2011). Also, in anoxic environments, certain NOB (*Nitrobacter*) can reduce nitrate coupled to the oxidation of organic compounds, which are internally stored as poly-β-hydroxybutyrate (PHB) (Freitag et al. 1987; Starkenburg et al. 2006).

A general overview on the nitrification (nitritation+nitratation) and denitrification conversions is given in Figure 1. 1.

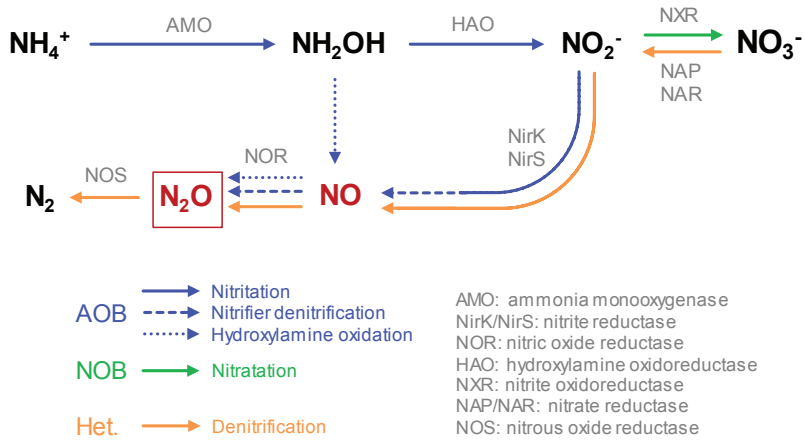

Figure 1. 1 Conventional nitrification (nitritation + nitratation) and denitrification transformations for nitrogen removal, including N_2O formation pathways (nitrifier denitrification and hydroxylamine oxidation by AOB, and incomplete denitrification by heterotrophs). The enzymes involved in each reaction are indicated.

1.4.2 Anaerobic ammonium oxidation (anammox)

Apart from the effluent quality, secondary objectives such as the reduction of energy consumption and greenhouse gas emission are gaining importance, aiming at future energy self-sufficient wastewater treatment plants (WWTPs) with zero carbon footprint (GWRC 2008). In this respect, more sustainable technologies to remove nitrogen from wastewater are based on the anaerobic ammonium oxidation (anammox) pathway, which is a short cut in the nitrogen cycle where ammonium is oxidized with nitrite as electron acceptor to nitrogen gas (Strous et al. 1998) (Eq. 1.4).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2H_2O \quad [1.4]$$

Strous et al. (2006) and Kartal et al. (2011) described the intermediates involved in the anammox catabolism, explaining how the anaerobic ammonium oxidation takes place. Nitrite is reduced to nitric oxide (Eq. 1.5), ammonium and nitric oxide are condensed to hydrazine (Eq. 6) and this is oxidized to nitrogen gas (Eq. 7). The electrons obtained from the later reaction are used during the two previous transformations.

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad [1.5]$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad [1.6]$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad [1.7]$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad [1.8]$$

Anammox bacteria are autotrophic microorganisms (i.e. grow on inorganic carbon, Eq. 1.4) and they are known to optimally thrive at 35 °C and pH 8. In general these bacteria have a slow growth rate and biomass productivity, as determined by Strous et al. (1998) (maximum growth rate (μ_{max}) = 0.0648 d^{-1}, doubling time of 11 d, and yield of 0.066 C-mol NH_4^+-mol^{-1}). However, in a recent study with *Candidatus* 'Brocadia fulgida' (one anammox species), these bacteria were characterized with the highest growth rate and yield ever reported (μ_{max} = 0.21 d^{-1}, doubling time of 3.3 d, and yield of 0.071 C-mol $NH4^+$-mol^{-1} (Lotti et al. 2014c)). The slow growth rate of anammox bacteria constitutes one of the main challenges for their implementation in wastewater facilities. On the other hand, the low growth yield also results in a low sludge production, disposal of which is one of the main cost factors in WWTPs.

1.4.3 Partial nitritation-anammox for wastewater treatment

In order to apply the anammox conversion for wastewater treatment, a preceding reaction yielding nitrite is required. Partial nitritation-anammox (also called deammonification) is one of the most common processes to apply the anammox pathway for wastewater treatment (Figure 1. 2A). During partial nitritation, half of the ammonium in the wastewater is oxidized to nitrite by AOB under aerobic conditions, avoiding the further oxidation to nitrate by NOB. Subsequently, anammox bacteria convert the remaining ammonium and the produced nitrite directly to nitrogen gas. Compared to conventional nitrification-denitrification over nitrate, partial nitritation-anammox is a more sustainable technology since requires up to 63% less aeration energy, produces less sludge, emits almost no CO_2 and can lead to up to 100% savings on external carbon addition due to the autotrophic nature of the process.

The realization of the partial nitritation and anammox conversions, under aerobic and anoxic conditions, respectively, can be established in **two**

separate reactors (two-reactor configuration) or in a single reactor (one-reactor configuration). The combined SHARON-anammox system (van Dongen et al. 2001) is an example of a two-reactor configuration. In one-reactor systems, both aerobic and anoxic conditions need to be established, which is typically realized in biofilm reactors or, more specifically, in granular sludge reactors. **Granular sludge** is a special type of biofilm in which biomass grows in compact, fast-settling aggregates (granules) without any carrier material. Compared to fixed biofilms growing on an inert surface (not moving bed biofilms), granular sludge presents a higher surface area and thus higher mass transfer area and conversion capacities. The studies carried out in chapters 2, 4 and 5 of this thesis involved granular biomass.

Biofilm/granular sludge reactors are particularly useful to grow slow growing organisms such as nitrifiers or anammox bacteria, since the SRT and HRT are uncoupled in these systems, allowing high hydraulic loads to the reactor without the washout of the biomass. Thus, biofilm / granular sludge reactors have a lower footprint than flocculent sludge technologies.

Moreover, inside the biofilm/granules diverse ecological conditions are present, allowing the coexistence of different microorganisms, such as aerobic ones (AOB) in the outer part of the biofilm/granules and anaerobic ones (anammox bacteria) in the inner anoxic part.

A common challenge for partial nitritation-anammox technology is the **suppression of NOB** to avoid the further oxidation of nitrite to nitrate and the competition with anammox bacteria for nitrite. Factors affecting the growth rates of AOB and NOB are typically used for this purpose. The aerobic ammonium oxidizers have higher affinity for oxygen than NOB, facilitating the suppression of the later bacteria by limiting the oxygen concentration in the system (Garrido et al. 1997). Moreover, AOB have higher growth rate than NOB at 30-35 °C, which can be used to washout the nitrite oxidizers by adjusting the sludge retention time (SRT) (Hellinga et al. 1998). The inhibitory effect of free ammonia (Vlaeminck et al. 2009) or free nitrous acid (Zhou et al. 2011) can be also used to limit the growth of NOB in partial nitritation-anammox systems, since these bacteria are more sensitive to free ammonia

and free nitrous acid than AOB. Tokutomi et al. (2010) found that high inorganic carbon concentrations could also be used to promote nitritation over nitratation. Limitation of inorganic carbon in the system hinders the grow of AOB, but does not affect the NOB development (Guisasola et al. 2007).

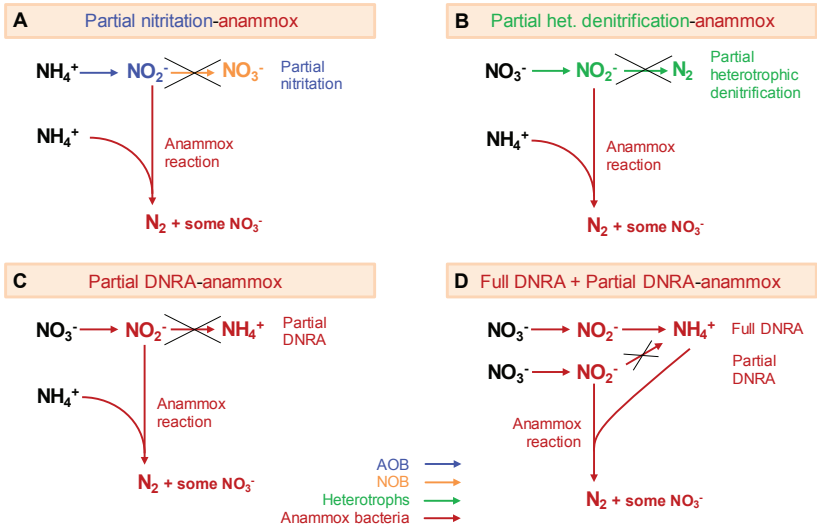

Figure 1. 2 Overview nitrite supply to carry out the regular anammox conversion: A) partial nitritation-anammox; B) partial heterotrophic denitrification-anammox; C) partial DNRA by anammox bacteria and anammox conversion with the nitrite formed during partial DNRA and external ammonium; D) full DNRA and partial DNRA, both by anammox bacteria, and anammox conversion with ammonium formed during full DNRA and nitrite formed during partial DNRA. The ammonium for reactions A, B and C is present in the wastewater while for reaction D is obtained from full DNRA by anammox bacteria.

In a two-reactor partial nitritation-anammox configuration, the partial nitritation process can be controlled independently from the anammox process by various control handles, as demonstrated for the SHARON partial nitritation process by (Volcke et al. 2006). In single stage biofilm/granular sludge reactors for partial nitritation-anammox, careful regulation of the oxygen concentration in the bulk liquid is required (Hao et al. 2002; Sliekers et al. 2002; Vlaeminck et al. 2010; Volcke et al. 2010). Other strategies keep the dissolved oxygen low and focus the control on the pH, since partial nitritation decreases the pH and the anammox reaction rises it (Wett 2007).

The partial nitritation-anammox technology was successfully implemented in the past decade at full-scale WWTPs for the treatment of sidestream (warm, ammonium-rich) wastewaters such as reject water from the anaerobic digester (Abma et al. 2010; Lackner et al. 2014; van der Star et al. 2007; Wett 2007). Sidestream wastewater conditions facilitate the successful of the partial nitritation-anammox technology: warm water (30-35 °C), low COD/N ratio (ammonium-rich), about equimolar amounts of alkalinity and ammonium, which allows the oxidation of half of the ammonium. However, the implementation of the anammox-based systems for mainstream (cold, low ammonium concentration, high organic carbon content) wastewater treatment presents some challenges, which are addressed in the next section. An overview of the characteristics of mainstream and sidestream wastewaters is presented in Table 1. 1.

Table 1. 1 Average characteristic composition, temperature and relative volume flow of sidestream and mainstream wastewaters. Composition data based on Lackner et al. (2014) for sidestream and Henze et al. (2008) for mainstream.

	Sidestream	Mainstream
Temperature (°C)	30-35	10-20
Relative volume flow (%)	1	99
Total COD (g COD m^{-3})	900	750
Ammonium (g N m^{-3})	750	45
COD/N (g COD/g N)	1-2	8-12

1.4.4 Anammox technology for mainstream wastewater treatment

Due to the advantages of anammox-based technologies compared to conventional nitrification-denitrification and the success in the sidestream of the WWTP, current scientific research is focused on the **implementation of anammox technology in the mainstream**, which volume flow accounts for 99% of the total volume flow of the WWTP (1% is reject water). Several studies have shown the potential advantages of implementing anammox-based technologies for mainstream wastewater treatment, aiming at energy positive WWTPs with zero carbon footprint (De Clippeleir et al. 2011; Jetten

et al. 1997; Kartal et al. 2010; Verstraete & Vlaeminck 2011). In these suggested configurations the organic carbon in the wastewater is concentrated or removed as sludge, by means of a concentrator or a high rate activated sludge (HRAS) system. Therefore, the chemical energy contained in the organic matter can be recovered in a later anaerobic digester as methane-rich biogas. For the nitrogen removal, anammox-based technologies are included after the organic carbon removal step.

The implementation of anammox-based systems in the mainstream of the WWTP is not straightforward and requires to overcome **challenging** conditions for anammox organisms and to accomplish the nitritation reaction. As opposed to the sidestream, the mainstream has lower temperature (ca. 15 °C instead of 30 °C), lower ammonium concentration (ca. 50 g N m^{-3} instead of 750 g N m^{-3}) and contains high amount of organic matter (see Table 1. 1). Also, the variability in flow rate and concentration in the mainstream pose a challenge to establish a stable and robust system treatment.

The feasibility of applying the anammox process for wastewater containing **low ammonium concentrations** and at **low temperatures** was demonstrated by various lab-scale studies (De Clippeleir et al. 2013; Hendrickx et al. 2014; Hendrickx et al. 2012; Lotti et al. 2014b). In those studies the feeding to the anammox reactor consisted of ammonium and nitrite. However, wastewater contains ammonium and the nitrite needs to be formed, which seems one of the main bottlenecks to establish partial nitritation-anammox during mainstream wastewater treatment. In a pilot-scale reactor treating the effluent from a HRAS, Lotti et al. (2015) found the limiting factor of the process the suppression of NOB for an effective nitrite formation needed for the anammox reaction.

The **suppression of NOB under mainstream conditions** is more difficult than under sidestream conditions. The nitrite oxidizers grow faster than AOB at low temperatures (Hellinga et al. 1998) and the low ammonium concentration does not favour the AOB activity due to their low affinity for ammonium, which hinders the growth of the aerobic ammonium oxidizers. With lower AOB activity the nitritation is limited and more oxygen is available

in the medium, which promotes the development of NOB. In general, the oxygen affinity of NOB is lower than the oxygen affinity of AOB. In a lab-scale reactor, partial nitritation was achieved at low temperature by controlling the oxygen concentration, but still excess of ammonium was needed (Isanta et al. 2015). Although it is accepted that the oxygen affinity of NOB is lower, some studies have found higher oxygen affinities for NOB than for AOB (Manser et al. 2005; Wu et al. 2015). In a modelling research of one-unit partial nitritation-anammox system under mainstream conditions, Pérez et al. (2014) found the ratio of the oxygen affinity constants of AOB and NOB a key factor for the suppression of the nitrite oxidizers, together with a relatively high ammonium concentration in the medium. In general, while the NOB could be hindered by an adequate oxygen and aeration control strategy, the low temperature and low ammonium concentration in the mainstream play an important role against the successful NOB suppression.

Apart from nitritation as a manner to supply nitrite to the anammox reaction, **partial heterotrophic denitrification** (i.e. reduction of nitrate to nitrite, see Figure 1. 2B) is also being investigated. Du et al. (2015) showed that the limitation of **organic carbon** and relatively short anoxic periods positively influence the accumulation of nitrite during partial denitrification. Higher concentrations of nitrate than nitrite were found to enhance the partial heterotrophic denitrification also (Yang et al. (2012). In this sense, the heterotrophic activity in established partial nitritation-anammox systems (low formation of nitrate) with small amount of organic compounds was shown positive to the overall nitrogen removal in the modelling study of Mozumder et al. (2014). Those authors found that the nitrate formed during the anammox conversion could be reduced to nitrite by heterotrophs and taken up by anammox bacteria, thus increasing the nitrogen removal efficiency in the system.

Regarding the presence of organic carbon compounds in the wastewater, it was recently discovered that anammox bacteria are able to perform **dissimilatory nitrate reduction to ammonium** (DNRA) with nitrite as intermediate and using volatile fatty acids (VFA) as electron donors

(organotrophic activity) (Guven et al. 2005; Kartal et al. 2007a; Kartal et al. 2007b; Winkler et al. 2012a) (Eq. 1.9). Anammox bacteria oxidize **organic carbon** (VFA) to CO_2 via acetyl-CoA (Russ et al. 2012), but without assimilation into biomass (Guven et al. 2005; Kartal et al. 2007b), hence keeping sludge production low.

$$NO_3^- + C_{org} \rightarrow (NO_2^-) \rightarrow NH_4^+ + CO_2 \quad [1.9]$$

DNRA by anammox bacteria has the potential to provide nitrite and/or ammonium for the well-known anammox transformation (from where the bacteria get energy to grow). Anammox bacteria could reduce nitrate to nitrite only (partial DNRA) and combine the formed nitrite with external ammonium present in the medium during the conventional anammox reaction (Figure 1. 2C). Besides, anammox bacteria could produce ammonium when performing full DNRA. The formed ammonium (through full DNRA) and the nitrite (through partial DNRA) could be combined in the regular anammox pathway to yield nitrogen gas (Figure 1. 2D). The organotrophic capacity of anammox bacteria could result beneficial during mainstream wastewater treatment since the bacteria could deal with the formation of nitrate (when NOB cannot be supressed) and some organic matter present. However, little is known about this pathway by anammox bacteria and what triggers this transformation over the heterotrophic DNRA (Eq. 1.9) and heterotrophic denitrification (Eq. 1.3), which also compete for nitrate and organic carbon.

1.5 Greenhouse gas emissions during wastewater treatment

During wastewater treatment, the greenhouse gases CO_2, N_2O and CH_4 are emitted. N_2O has a global warming potential of 298-CO_2 equivalents over 100 year horizon, while CH_4 accounts for 34-CO_2 equivalents (IPCC 2013). Therefore, even small emissions of N_2O and CH_4 may have a strong environmental impact and contribute significantly to the carbon footprint of the WWTP. The total greenhouse gas emissions from the waste and wastewater sector accounted for the 3% of the total anthropogenic greenhouse gas emissions in 2010 (1.4 $GtCO_2eq$) (IPCC 2014a). Within these,

the greenhouse gas emissions from wastewater handling estimated by the IPCC (2014a) accounted for 54% (781 $MtCO_2eq$). CH_4 was the main greenhouse gas from the wastewater sector (667 $MtCO_2eq$), followed by N_2O (108 $MtCO_2eq$). However, these numbers should be taken with caution, since actual measurements in wastewater treatment facilities show a high variability (Daelman et al. 2013a). For instance for N_2O, Kampschreur et al. (2009b) reported an emission range from 0 to 14.6% of the total ammonium removed and Law et al. (2012b) from 0 to 25%.

1.5.1 CO_2 emissions

The main source of CO_2 emissions during wastewater treatment is the combustion of fossil fuels to generate energy and electricity required in the plant. For a WWTP with primary settling, activated sludge system and anaerobic digestion, Siegrist et al. (2008) calculated a net energy consumption of 0.044 kWh $person^{-1}$ d^{-1}. Aeration, mainly needed for organic matter and nitrogen removal, together with pumping are the main energy sinks during wastewater treatment.

The respiration of organic matter present in the wastewater by the bacterial communities produces also CO_2. These CO_2 emissions are typically considered biogenic (i.e. its origin comes from the natural carbon cycle) and thus assumed to not contribute to the global warming (EPA 2014). However, specific studies have shown that around 3-14% of the total organic carbon entering in the WWTP has a fossil origin, which should be taken into account when accounting for the CO_2 emissions from wastewater treatment (Law et al. 2013a).

1.5.2 N_2O emissions

N_2O can contribute significantly to the carbon footprint of the WWTP, e.g. accounting for the 78% of the total greenhouse gas emissions (CO_2 equivalents) as reported by Daelman et al. (2013b). Because of its high global warming potential (298-CO_2 equivalents) and its negative influence on the ozone layer depletion (Ravishankara et al. 2009; Revell et al. 2015), a lot of

attention is paid to the reduction of N_2O emissions from wastewater treatment.

N_2O can be produced during biological nitrogen removal from wastewater during nitritation (Eq. 1.1), heterotrophic denitrification (Eq. 1.3) or by chemical reactions (Kampschreur et al. 2009b). An overview of the biological pathways producing N_2O is shown in Figure 1. 2.

Nitritation

Although ammonium oxidizing archaea (AOA) and some heterotrophic organisms are able to carry out ammonium oxidation, the main contributors to N_2O formation during nitritation are AOB. Two main routes are known to yield N_2O by AOB during nitritation: the oxidation of hydroxylamine (NH_2OH), which is enhanced by high ammonium oxidation rates (Wunderlin et al. 2012); and the reduction of nitrite to nitric oxide (NO) (an intermediate) and N_2O (nitrifier denitritrification), which is favoured by limiting oxygen conditions (Goreau et al. 1980; Tallec et al. 2006), since this promotes the use of nitrite as electron acceptor instead of oxygen, and the presence of nitrite (Colliver & Stephenson 2000) (see Figure 1. 2).

Heterotrophic denitrification

Since N_2O is an intermediate during heterotrophic denitrification, this gas can be emitted during an incomplete denitrification. Also, the denitrification can act as a sink for N_2O removal due to the action of the enzyme NOS (see Figure 1. 2). Heterotrophic denitrification can contribute significantly to N_2O emissions during biological nitrogen removal in the presence of oxygen (Otte et al. 1996), because of the inhibition of the enzyme NOS by oxygen, or when there is not enough organic carbon in the wastewater to complete the denitrification (Chung & Chung 2000).

Chemical reactions

Under high nitrite concentrations, NO can be produced chemically from the disproportionation of nitrous acid (Udert et al. 2005) or from the reaction

between nitrite and Fe (II) (Kampschreur et al. 2011) leading to the subsequent formation of N_2O.

N_2O emissions from anammox conversion?

Given that N_2O is not involved in the anammox metabolism (reactions 1.5-1.7) (Kartal et al. 2011), it is accepted that anammox bacteria do not contribute to N_2O emissions. However, NO is an intermediate during the formation of nitrogen gas during the anammox reaction (Eq. 1.5 and 1.6), which could lead to N_2O emissions in side reactions, as suggested by Lotti et al. (2014c). In an almost pure anammox culture (98 ±1%), those authors found N_2O emissions of 0.056 ± 0.02% of the total nitrogen load, which could be explained by chemical formation and/or by other bacterial conversions that can reduce NO to N_2O. Although anammox bacteria could indirectly contribute to N_2O production in this way, this contribution is considered negligible in most practical cases.

1.5.3 CH_4 emissions

CH_4 is produced in the sewer system (Guisasola et al. 2008; Liu et al. 2015) and in those zones where anaerobic conditions prevail, including sludge thickeners and buffer and storage tanks related to anaerobic digesters (Daelman et al. 2012). CH_4 emissions in sewers present high variations, but it was estimated that they could contribute significantly to the carbon footprint of the WWTP by 18% (Liu et al. 2015). Important CH_4 emissions can occur during anaerobic sludge treatment. Plants including anaerobic digesters have shown higher total CH_4 emissions than plants without these units. In this respect, not only direct CH_4 emissions to the atmosphere should be taken into account, but also the dissolved CH_4 in the reject water that can escape to the atmosphere during downstream processing. Other sludge handling treatments such as the sludge drying lagoons in tropical and subtropical climates also have potentially important contributions to CH_4 emissions (Pan et al. 2016).

In contrast to N_2O monitoring campaigns, few studies have been dedicated to determine actual CH_4 emissions from WWTPs. The CH_4 emission factors

found in literature in terms of kg CH_4 kg $COD_{influent}^{-1}$ vary significantly depending on the sampling strategy. Emissions factors of 0.08% and 0.16% were found by Wang et al. (2011) and Czepiel et al. (1993), respectively, using grab sampling methodologies. The emissions factors found during online monitoring campaigns are significantly higher, as reported by Daelman et al. (2012) in a long-term campaign in a WWTP in the Netherlands (1.13%, kg CH_4 kg $COD_{influent}^{-1}$) or in the more recent study of Lorenzo-Toja et al. (2016) that includes online CH_4 measurements in two WWTP in Spain (1.05% and 1.15%, kg CH_4 kg $COD_{influent}^{-1}$).

1.6 How could greenhouse gases be reduced through nitrogen removal systems from wastewater?

CO_2 emissions can be reduced by implementing **anammox-based technologies** instead of conventional nitrification-denitrification over nitrate to remove nitrogen from wastewater, since substantial savings in aeration can be achieved. It was estimated by Kartal et al. (2010) that the energy demand in a WWTP with reject water treated by anammox-based systems could be 0.021 kWh $person^{-1}$ d^{-1} compared to the 0.044 kWh $person^{-1}$ d^{-1} in a conventional WWTP, and the authors also claimed a generation of energy (0.024 kWh $person^{-1}$ d^{-1}) when considering a configuration with anammox-based technology in the main line of the WWTP. However, while anammox-based technologies, such as partial nitritation-anammox, were successfully implemented for the treatment of reject water (section 1.3.3), the **implementation in the mainstream** of the WWTP is still a challenge (section 1.3.4), which main bottleneck is an effective nitrite supply to the anammox reaction (Lotti et al. 2015).

It is known that technologies relying on partial nitritation-anammox result in a significant reduction of CO_2 emissions. However, the impact in terms of **N_2O emissions** of this technology compared to conventional nitrogen removal techniques remains unclear. Especially for **reject water**, N_2O emissions could result significantly important. The high ammonium concentration can lead to high ammonium consumption rates, which is known to enhance N_2O

production (Wunderlin et al. 2012). Also, nitrite formation and accumulation during partial nitritation can promote N_2O emissions (Okabe et al. 2011) and the limiting oxygen (Goreau et al. 1980) or transient from anoxic to aerobic (Yu et al. 2010) conditions to prevent the oxidation of nitrite to nitrate by NOB. The **evaluation of operational conditions** through online full-scale measurements to capture the actual N_2O dynamics is needed. Thereby, appropriate control strategies that may lead to effectively reduce N_2O emissions from partial nitritation-anammox systems could be established.

Regarding **mainstream wastewater** treatment, **N_2O emissions** were quantified in several studies (Ahn et al. 2010b; Czepiel et al. 1995; Foley et al. 2010). However, few researches have addressed deeply specific conditions that trigger N_2O emissions. Some studies focussed on the effect of oxygenation (Kimochi et al. 1998; Tallec et al. 2006), aeration (Rodriguez-Caballero et al. 2015) or the seasonal variability (Daelman et al. 2015), but without measuring the important intermediate NO (see Figure 1. 2). In general it is known that nitrite enhances N_2O emissions (Colliver & Stephenson 2000). During municipal wastewater treatment, the accumulation of nitrite may happen depending on the operating conditions and loading rates at certain moments, and thus raising N_2O emissions (Daelman et al. 2015). Moreover, the suppression of NOB for the implementation of anammox-based systems for municipal wastewater treatment may lead to the accumulation of nitrite. In a lab-scale granular sludge reactor, Lotti et al. (2014a) assessed the implementation of partial nitritation-anammox under mainstream conditions, achieving the suppression of NOB by establishing an aerobic/anoxic regime where nitrite accumulated during the aerobic phase. Therefore, understanding the factors that lead to N_2O emissions during the treatment of low strength ammonium wastewaters, such as **nitrite accumulation**, would lead to the minimization of this greenhouse gas and would contribute to more sustainable nitrogen removal systems from municipal wastewater treatment.

Apart from the high ammonium concentration, that could be removed by anammox-based technologies in granular sludge systems, reject water

contains **dissolved CH_4**. It was recently found that anaerobic methane oxidation can take place coupled to denitrification (Raghoebarsing et al. 2006). Denitrifying anaerobic methane oxidation (damo) can be carried out by bacteria (damoB), namely *Candidatus* 'Methylomirabilis oxyfera', which couple CH_4 oxidation to nitrite (NO_2^-) reduction (Ettwig et al. 2010; Luesken et al. 2011) (Eq. 1.10). M. oxyfera oxidizes CH_4 in a very particular way. This bacterium intracellularly produces oxygen that is used for the oxidation of CH_4 as in the typical aerobic methane oxidation. Also, some archaea were found to perform damo process (damoA) such as *Candidatus* 'Methanoperedens nitroreducens', which utilizes nitrate (NO_3^-) as electron acceptor (Haroon et al. 2013) (Eq. 1.11).

damoB $$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad [1.10]$$

damoA $$2CH_4 + 8NO_3^- \rightarrow 2CO_2 + 8NO_2^- + 4H_2O \quad [1.11]$$

Simultaneous ammonium and CH_4 removal by anammox and damo microorganisms seems a beneficial technology for more sustainable WWTP. So far, the feasibility of such a system was demonstrated under anoxic conditions (i.e. not considering the nitritation step) in lab-scale systems (Hu et al. 2015; Shi et al. 2013) and modelling approaches (Chen et al. 2014; Winkler et al. 2015). However, the application of damo processes in aerobic partial nitritation-anammox granular sludge systems was not evaluated, which potential success could contribute to reduce the carbon footprint from WWTP.

1.7 Outline of the thesis

This thesis deals with the reduction of the greenhouse gases CO_2, N_2O and CH_4 during nitrogen removal from wastewater treatment, for both mainstream and sidestream wastewater, applying different methodologies, as detailed in Table 1. 1.

Table 1. 1 Overview of the greenhouse gases and type of wastewater studied in each chapter as well as the methodology applied.

Chapter		Main greenhouse gas studied	Type of wastewater	Methodology
2	Implementation of anammox technology in the mainstream	CO_2	Mainstream (low strength ammonium, cold)	Laboratory experiments
3	Effect of nitrite on the N_2O and NO production	N_2O		
4	Effect of aeration regime on N_2O emissions		Sidestream (high strength ammonium, warm)	Full-scale monitoring campaign
5	Integration of CH_4 removal in aerated anammox-based reactors	CH_4		Mathematical modelling and simulation

Chapter 2 is dedicated to the potential application of nitrate reduction by organotrophic anammox bacteria (i.e. reduction of nitrate to nitrite coupled to the oxidation of organic carbon by anammox bacteria, together with the conventional anammox reaction) for the implementation of anammox-based technologies in the mainstream of the WWTP. Laboratory batch tests with anammox biomass as such and anammox and heterotrophic bacteria were carried out with different feeding conditions (ammonium, nitrate, nitrite and acetate). Also, a sequencing batch reactor was operated with semi-continuous acetate and nitrate feeding. Factors triggering the prevalence of the organotrophic nitrate reduction by anammox bacteria over heterotrophic nitrate conversions were studied, gathering valuable knowledge for the potential implementation of this innovative technology. Also, a scheme involving the nitrate reducing capacity of anammox bacteria was suggested, which could overcome current bottlenecks for the implementation of anammox-based technologies under mainstream conditions.

Certain operational conditions as well as the implementation of anammox-based technologies may lead to the accumulation of nitrite during municipal wastewater treatment. **Chapter 3** analyses the effect of nitrite accumulation on the N_2O and NO production in a nitrifying lab-scale system treating low ammonium strength wastewater. N_2O and NO in the off-gas of the reactor

were monitored online while supplying nitrite shock additions to the reactor and while step-wisely increasing the nitrite concentration. The insights obtained on the responsible bacterial pathways producing N_2O and NO, together with the correlations between these gases and nitrite, contribute to the potential reduction of N_2O and NO during mainstream wastewater treatment with momentary nitrite accumulation.

Chapter 4 sheds a light on the parameters influencing N_2O emissions during nitrogen removal from high ammonium concentrated sidestream wastewater, such as reject water. More particular, the effect of the aeration regime on the N_2O emissions was investigated for a full-scale granular sludge partial nitritation-anammox reactor treating reject water. A monitoring campaign was carried out, gathering online N_2O emissions in the off-gas of the reactor under normal reactor operation and during a dedicated experiment.

Besides high ammonium concentrations, reject water contains dissolved CH_4. In **Chapter 5**, the potential integration of methane removal in aerobic granular sludge reactors performing partial nitritation-anammox was investigated. A model was set up including anammox bacteria, anaerobic methane oxidizing bacteria and archaea, aerobic methane oxidizing bacteria, besides AOB, NOB and heterotrophs. The substrate competition among these communities was evaluated through simulation at different ammonium and oxygen concentrations. Also, the possible CH_4 stripping due to the aeration was assessed and strategies to minimize it are suggested. The outcome of this chapter contributes to the implementation of this combined methane-nitrogen removal technology.

Chapter 6 gives concluding remarks on how the findings obtained in this thesis contribute to the minimization of CO_2, N_2O and CH_4 emissions and thus to the reduction of the carbon footprint from urban WWTP.

2

Evaluating the potential for nitrate reduction through DNRA by anammox bacteria for municipal wastewater treatment

Abstract

Anammox bacteria can perform dissimilatory nitrate reduction to ammonium (DNRA) with nitrite as intermediate coupled to the oxidation of volatile fatty acids. The objective of this chapter is to gain understanding on this metabolic pathway. Batch tests with anammox as such and anammox and heterotrophic bacteria showed the capacity of *Candidatus* 'Brocadia fulgida' to perform DNRA coupled to the anammox reaction (DNRA-anammox) even if the culture was not previously adapted to volatile fatty acids. The energy usage of the anammox driven DNRA pathway is not unravelled yet but from thermodynamic calculations it can be stated that low COD/N influent ratios favour the DNRA-anammox transformation over heterotrophic conversions since more free energy is gained (1984 kJ acetate-mol^{-1} during DNRA-anammox vs. 797 during heterotrophic denitrification). This paper further proposes the implementation of an innovative nitrogen removal system in which the nitrate produced by nitrite oxidizing bacteria and/or anammox bacteria is converted during DNRA-anammox pathway, resulting in sustainable nitrogen removal from municipal wastewater.

Chapter redrafted after:

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2.1 Introduction

The anaerobic ammonium oxidation (anammox) conversion comprises the oxidation of ammonium with nitrite as electron acceptor to nitrogen gas under anoxic conditions. The implementation of the anammox process needs a preceding reaction in which half of the ammonium in the wastewater is oxidized to nitrite by ammonium oxidizing bacteria (AOB) (partial nitritation). For the success of this partial nitritation-anammox system (Figure 2. 1A), the further oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB) must be prevented. Compared to conventional nitrification-denitrification, partial nitritation-anammox requires up to 63% less aeration energy, produces less sludge, emits almost no CO_2 and leads to up to 100% savings on addition of external organic carbon (Siegrist et al. 2008).

Anammox-based processes were successfully implemented at several full-scale plants to treat warm ammonium-rich reject water from anaerobic digesters (sidestream) (Abma et al. 2010; Lackner et al. 2014; van der Star et al. 2007; Wett 2007). Due to the advantages of this technology in the sidestream, current scientific research is focused on the implementation of anammox technology in the mainstream of the WWTP (Kartal et al. 2010; Lotti et al. 2014b). However, different challenges need to be overcome for successful application of this process in mainstream. As opposed to the sidestream, the mainstream has lower temperature (down to ca. 10 °C instead of 30 °C), stronger effluent requirements, lower ammonium concentrations (ca. 50 g N m^{-3} instead of 750 g N m^{-3}), and contains higher amount of biodegradable organic matter (higher COD/N ratio). Anammox bacteria are known to optimally thrive at 35 °C, but different studies have shown that an implementation of the anammox process at low temperature is also feasible (Hendrickx et al. 2014; Lotti et al. 2014a; Vazquez-Padin et al. 2009). In these studies the nitrite was added directly in the anammox reactor. Nevertheless, in practice, the nitrite needs to be formed. In this regard, Lotti et al. (2015) tried to establish partial nitritation-anammox in a pilot-scale reactor to treat a mainstream effluent and found that the formation of nitrite (and suppression of NOB) was the limiting factor of the process. Therefore, one of

the main major bottleneck for the application of anammox-based technologies in the mainstream seems to be an effective nitrite supply to the anammox pathway. Although NOB have a lower affinity for oxygen than AOB, the presence of NOB cannot be out ruled in the main line if operated under oxygen limiting conditions (Pérez et al. 2014). The difficulty to deselect against NOB can be also explained by growth rates. At lower temperature, it is not possible to outcompete NOB based on sludge retention time (SRT) such as it is commonly done in a two stage system, which is taking advantage of the faster growth rate of AOB at high temperatures by operating the system at SRT below the minimal required growth rate of NOB (Hellinga et al. 1998). However, at low temperatures (such as in mainstream) NOB possess a faster growth rate than AOB and therefore NOB cannot be out selected based on SRT control and hardly controlled by oxygen limitations either.

Beside nitritation as a manner to provide nitrite to the anammox reaction during municipal wastewater treatment, other approaches concern the partial denitrification until nitrite only (Figure 2. 1B). For the heterotrophic denitrification, the limitation of organic carbon and relatively short anoxic periods positively influence the accumulation of nitrite (Du et al. 2015). In addition, recent studies have shown that low amount of organic compounds in partial nitritation-anammox systems could improve the overall nitrogen removal by reducing nitrate to nitrite by heterotrophic denitrification that could be taken up by anammox microorganisms (Mozumder et al. 2014). However, the limitation of organic compounds to achieve incomplete denitrification to nitrite could enhance N_2O formation by heterotrophs, which would decrease the sustainability of the process (see section on N_2O formation 1.5.2).

Regarding the presence of organic carbon compounds in the wastewater, it was recently discovered that anammox bacteria are able to perform dissimilatory nitrate reduction to ammonium (DNRA) with nitrite as intermediate using volatile fatty acids (VFA) as electron donor (organotrophic activity) (Guven et al. 2005; Kartal et al. 2007a; Kartal et al. 2007b; Shu et al. 2015; Winkler et al. 2012a). The VFA are oxidized to CO_2 via acetyl-CoA (Russ

et al. 2012) but are not assimilated into biomass (Guven et al. 2005; Kartal et al. 2007b), hence keeping sludge production low. In some experiments, DNRA by anammox bacteria was reported to occur coupled to the anammox transformation (from where the bacteria get energy to grow) in two ways: converting the nitrate to nitrite only (partial DNRA) and combining the nitrite formed with external ammonium present in the environment to yield nitrogen gas during the anammox reaction (Figure 2. 1C). Also, anammox bacteria were shown to reduce part of the nitrate to the intermediate nitrite only (partial DNRA) and part to ammonium, the end product of DNRA (full DNRA) and then to combine both nitrite and ammonium formed (Figure 2. 1D) (Kartal et al. 2007a).

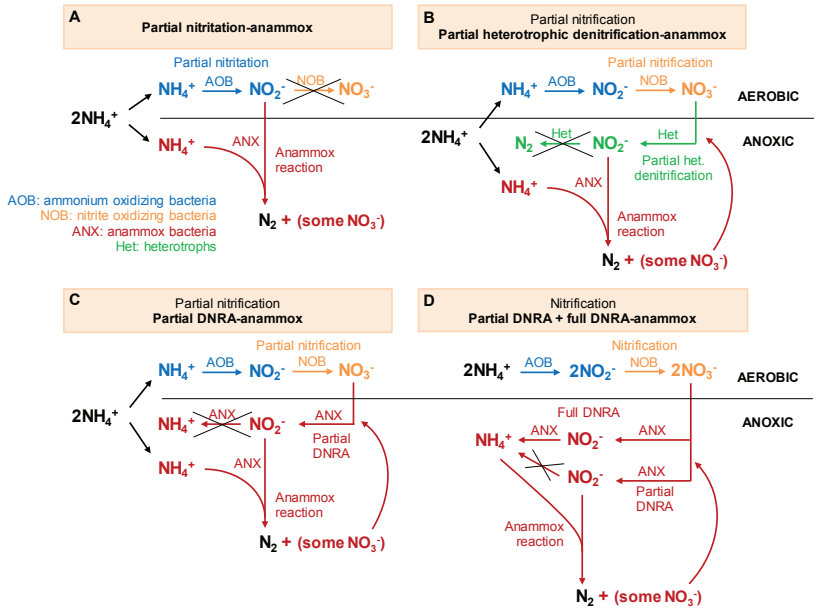

Figure 2. 1 Comparison of different nitrogen removal configurations involving the anammox process: A) partial nitritation-anammox; B) partial nitrification of the ammonium + partial heterotrophic denitrification-anammox; C) partial nitrification of the ammonium + partial DNRA (reduction of nitrate to nitrite only by anammox bacteria) and anammox reaction with the nitrite formed and the remaining ammonium available; D) nitrification of the ammonium + partial DNRA, full DNRA and anammox reaction with nitrite formed during partial DNRA and ammonium formed during full DNRA.

The main challenge to establish DNRA by anammox bacteria is their out-competition by heterotrophic bacteria, which compete for nitrate and acetate

and which have a much faster growth rate. In general, for heterotrophs, DNRA pathway is promoted over denitrification under nitrate limiting conditions (i.e. high COD/N ratios) and when nitrate (and not nitrite) is the main electron acceptor present (Kraft et al. 2014; van den Berg et al. 2015). However, little is known about the conditions that promote anammox bacteria performing DNRA over the heterotrophic utilization of carbon. Some anammox species, enriched in lab-scale reactors, were found to effectively outcompete heterotrophs for propionate (*Candidatus* 'Anammoxoglobus propionicus', Kartal et al. (2007b)), and acetate (Candidatus 'Brocadia fulgida', Kartal et al. (2008)) in the presence of ammonium, but the conclusions remained descriptive.

The main objective of this study was to gain understanding on the DNRA pathway of anammox bacteria. The bacterial populations were determined in three case-studies and the factors promoting the DNRA by anammox bacteria over heterotrophic transformations evaluated. Thermodynamic calculations were determined to assess the energy gained per mole of substrate and electron transferred during different transformations involving the conversion of nitrate and acetate. An innovative and sustainable system to remove nitrogen from municipal wastewater is also proposed.

2.2 Materials and methods

Batch tests were conducted using anammox biomass from a full-scale granular sludge reactor (Case A, described in section 2.2.1) and sludge from a sequencing batch reactor (SBR) operated with anammox bacteria and phosphate accumulating organisms (PAOs) among other communities (Case B, described in section 2.2.2). The third set-up consisted of an anammox SBR operated under a semi-continuous exposure to organic carbon (acetate) (Case C, described in section 2.2.3) (see Table 2. 1).

2.2.1 Batch tests with anammox granular sludge from a full-scale WWTP (Case A)

A series of 6 different batch tests were performed using biomass taken from the anammox granular sludge reactor from the WWTP of Rotterdam. The biomass was first washed with tap water and then used in each batch test, which were conducted in a 2 L bioreactor. Before each test, nitrogen gas was supplied to the bioreactor for 30 min to ensure anoxic conditions. Different initial substrate concentrations were added in each batch test with a syringe through a septum (Table 2. 1, Case A), together with a mineral medium according to van de Graaf et al. (1996) and Dapena-Mora et al. (2004) (Appendix, Table A2. 1 and Table A2. 2). Inorganic carbon was provided in the mineral medium in excess as $KHCO_3$ (1 g $KHCO_3$ L^{-1}). The temperature was controlled at 30 ± 1 °C by using a heating water jacket connected to a circulating bath and the pH was not controlled. Methanol (MeOH) was added in some batch tests to assess the potential inhibitory effect of the alcohol on the bacterial activities. Liquid samples were collected at different times along the batch tests to determine the consumption rates.

Table 2. 1 Initial substrate concentrations for the batch tests with biomass from a full-scale anammox granular sludge reactor (Case A), from a bubble column SBR containing anammox bacteria and PAOs among other communities (Case B), and feeds used during the operation of the SBR of Case C.

		NH_4^+ (g N m^{-3})	NO_3^- (g N m^{-3})	NO_2^- (g N m^{-3})	Acetate (g COD m^{-3})	MeOH (g COD m^{-3})	COD/ NO_3^--N[1] -
Case A							
Batch test							
A.1	**NH_4^+ + NO_2^-**	200		200			
A.2	**NH_4^+ + NO_2^- + MeOH**	200		200		576	
A.3	**NH_4^+ + NO_3^- + acetate**	200	100		460		4.6
A.4	**NH_4^+ + NO_3^- + acetate + MeOH**	100	50		230	576	4.6
A.5	**NO_3^- + acetate**		50		230		4.6
A.6	**NH_4^+ + NO_3^- + acetate (no HCO_3^-)**	100	50		230		4.6
Case B							
Batch test							
B.1	**NH_4^+ + NO_2^-**	147		50			
B.2	**NH_4^+ + NO_2^- + MeOH**	147		50		240	
B.3	**NH_4^+ + NO_3^- + acetate**	147	50		76		1.5
B.4	**NO_3^- + acetate**		50		76		1.5
B.5	**NO_3^- + acetate + MeOH**		50		76	240	1.5
B.6	**NH_4^+ + NO_2^- + acetate**	147		50	76		1.5
B.7	**NO_2^- + acetate**			50	76		1.5
B.8	**NO_2^- + acetate + MeOH**			50	76	240	1.5
Case C							
'Anammox regime'							
Feed C.1	**NH_4^+ + NO_2^-**	125-200		100-200			
'Regime under semi-continuous exposure to acetate'							
Feed C.2	**NH_4^+ + NO_3^- + acetate (no HCO_3^-)**	50-100	50-100		50-100		
Feed C.3	**NH_4^+ + NO_2^- (no HCO_3^-)**	50-200		50-200			

[1] Initial substrate ratio COD/NO_3^--N or COD/NO_2^--N in the batch test

2.2.2 Batch tests with anammox and heterotrophic bacteria (Case B)

A series of 8 batch tests were performed with granular biomass collected from a bubble column SBR operated under intermittent anoxic/aerobic conditions, carrying out nitritation-anammox and organic carbon removal via DNRA process as described in Winkler et al. (2012a). The difference in the present study and in Winkler et al. (2012a) if that the reactor operation was changed to a two-fold higher acetate/nitrogen in the influent, from 0.5 (in Winkler et al. (2012a)) to 0.9 (in the present study). The biomass was collected from the SBR after the aerated phase and washed with tap water. All batch tests were

completed in glass flasks containing 250 mL, in which nitrogen gas was supplied for 25 minutes prior to the start of the tests to ensure anoxic conditions. Each test was induced with a different set of substrates as listed in Table 2. 1 (Case B) and same mineral medium and trace elements as used during the operation of the reactor (Winkler et al. 2012a). Temperature and pH were not controlled during the batch tests. As in the batch tests of Case A (section 2.2.1), MeOH was added in some tests to assess a potential inhibitory effect in the bacterial activities. Samples were taken over time during each batch test.

2.2.3 Sequencing batch reactor under semi-continuous acetate supply (Case C)

A SBR of 2.3 L was inoculated with anammox granular sludge from the CANON reactor from the wastewater treatment plant of Amersfoort (The Netherlands). The reactor was operated in 6 hour cycles with two different regimes (Figure 2. 2).

'Anammox regime' (1 month): 150 min of continuous feeding + mixing (Feed C.1 (Table 2. 1, Case C), $NH_4^+ + NO_2^-$), 120 min of mixing, 60 min settling and 30 min decanting.

'Regime under semi-continuous exposure to acetate' (1 month). The bacteria were intermittently fed with acetate as the only carbon source (no inorganic carbon was added). Phase with acetate: 90 min of continuous feeding + mixing (Feed C.2 (Table 2. 1, Case C), $NH_4^+ + NO_3^-$ + acetate), 30 min of mixing. Phase without acetate: 120 min of continuous feeding + mixing (Feed C.3 (Table 2. 1, Case C), $NH_4^+ + NO_2^-$) and 30 min of mixing. Then a settling period of 60 min and decanting of 30 min.

	Phase	Feeding + mixing	Mixing	Settling	Decant.
Anammox regime	**Feed**	$NH_4^+ + NO_2^- + HCO_3^-$	-	-	-
	t (min)	150	120	60	30

	Phase	Feeding + mixing	Mixing	Feeding + mixing	Mixing	Settling	Decant.
Regime under semi-continuous exposure to acetate	**Feed**	$NH_4^+ + NO_3^-$ + Acetate	-	$NH_4^+ + NO_2^-$	-	-	-
	t (min)	90	30	120	30	60	30

Figure 2. 2 Cycle operation during the 'Anammox regime' and 'Regime under semi-continuous exposure to acetate' of the SBR of Case C.

For both regimes, 1 L of medium was fed in each cycle. The feeding pump for the 'Anammox regime' was set at 6.67 mL min^{-1}, while the feeding flow rate of the 'Regime under semi-continuous exposure to acetate' was 4.76 mL min^{-1}. The modifications on the composition of the different feeds used are summarized in Table 2. 1, Case C. In addition to the compounds of these feeds, a mineral medium was supplied (Appendix, Table A2. 1 and A2. 2). The effect of MeOH was not assessed during the operation of the SBR in Case C to avoid the damage to the bacterial population. Although the reactor was inoculated with granular sludge, the granules disintegrated after 2 days of operation due to the mixing applied to keep homogenous conditions (magnetic stirrer). The temperature was controlled at 30 ± 1 °C by using a heating water jacket connected to a circulating bath and the pH was kept at 7.4 ± 0.4.

This reactor was operated to first determine the anammox performance during the 'Anammox regime', and secondly to assess the effect of the absence of inorganic carbon when feeding the reactor intermittently with organic carbon (acetate) during the 'Regime under semi-continuous exposure to acetate'. No inorganic carbon was supplied during this regime and two different COD/NO_3^--N ratios in the influent were applied (1.1 and 0.6 g COD (g N)$^{-1}$ to assess the ammonium, nitrate and acetate consumption rates. The specific substrate concentrations used are indicated below:

Ratio 1.1: Acetate=115 g COD m^{-3}; nitrate=104 g N m^{-3}

Ratio 0.6: Acetate=42 g COD m^{-3}; nitrate=67.5 g N m^{-3}

Liquid samples were taken during both regimes along the cycles to evaluate the bacterial performance.

2.2.4 Analytical methods and FISH analysis

The total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods (APHA 1998). Liquid samples were taken and immediately filtered through disposable Millipore filters (0.45 µm pore size). Ammonium, nitrate, nitrite and COD (acetate) were determined with standard test kits Hach-Lange and Macherey-Nagel for Cases A and C. The nitrogen compounds in Case B were analysed with QuikChem 8500 Flow Injection Analysis (FIA) from Lachat Instruments and the acetate using a High Performance Liquid Chromatography (HPLC) system.

Fluorescence in situ hybridization (FISH) and slicing was performed on the granular biomass from the bubble column SBR for Case B according to the method proposed by Winkler et al. (2011) in order to observe the population spatial distribution inside the granules. Also, FISH was accomplished on the flocculent biomass from the anammox SBR operated with a transient exposure to acetate (Case C). The oligonucleotide probes used are listed in Table 2. 2. Note that NOB probes were not used.

Table A2. 2 Oligonucleotide probes used in this study for FISH analysis.

Probe	Specificity	Reference
EUB 338	Most bacteria	(Amann et al. 1990)
Amx 368	All anammox bacteria	(Schmid et al. 2003)
Amx 820	*Ca.* Brocadia and *Ca.* Kuenenia	(Schmid et al. 2000)
BFU 613	*Ca.* Brocadia fulgida	(Kartal et al. 2008)
NSO 190	All AOB	(Mobarry et al. 1997)
NSO 1225	All AOB	(Mobarry et al. 1997)

2.2.5 Calculation of the specific substrate consumption rates

The specific substrate consumption rates (ammonium (r_{NH4}), nitrate (r_{NO3}), nitrite (r_{NO2}) and acetate (r_{COD})) during the batch tests of Cases A and B were calculated through linear regression based on the change of the substrate amount over time. The specific consumption rates for the different substrates in Case C were calculated through mass balances accounting for the amount of substrate added between two different times (t_1 and t_2 (h)) along the cycle of the reactor (Eq. 2.1).

$$r_x = \frac{M_x(t_1) + C_{x\_inf} \cdot Q \cdot (t_2 - t_1) - M_x(t_2)}{(t_2 - t_1) \cdot VSS} \quad [2.1]$$

Where:

r_x: specific consumption rate of substrate *x* (mg *x* (g *VSS*)$^{-1}$ h^{-1}).

M_x: mass of substrate *x* (mg *x*).

C_{x_inf}: concentration of substrate *x* in the influent (mg *x* L^{-1}).

Q: feeding flow rate (L h^{-1}).

VSS: volatile suspended solids (g *VSS*).

2.3 Results and discussion

Nitrate reduction by heterotrophs and anammox bacteria was evaluated in three case studies: batch tests with anammox biomass from a full-scale reactor (Case A), batch tests with anammox and heterotrophic biomass from a SBR (Case B) and sequencing batch operation of a bioreactor with anammox biomass (Case C) (see Table 2. 1 for more information on the conditions of each Case study). The results from bacterial population determination are presented in section 2.3.1, and the anammox and nitrate reduction activities assessed in the absence and presence of organic carbon (acetate) are described in section 2.3.2. The factors influencing the acetate and nitrogen transformations among heterotrophs and anammox bacteria are discussed in section 2.3.3 and 3.4 (i.e. competition heterotrophs vs. anammox bacteria). Finally, a scheme to apply the combined partial DNRA-anammox by anammox bacteria is presented in section 2.3.5.

2.3.1 Bacterial populations

FISH analysis were accomplished to determine the dominant bacterial populations (Figure 2. 3). The anammox species found in the three cases of this study was *Candidatus* 'Brocadia fulgida', of which the ability to carry out DNRA and outcompete heterotrophs for volatile fatty acids (VFA) was demonstrated in previous studies (Kartal et al. 2008; Winkler et al. 2012a; Winkler et al. 2012b). Concerning their relative abundance, anammox

bacteria were the dominant organisms present in Case A (data not shown) and in Case C (Figure 2. 3A), with relatively little amount of other microorganisms.

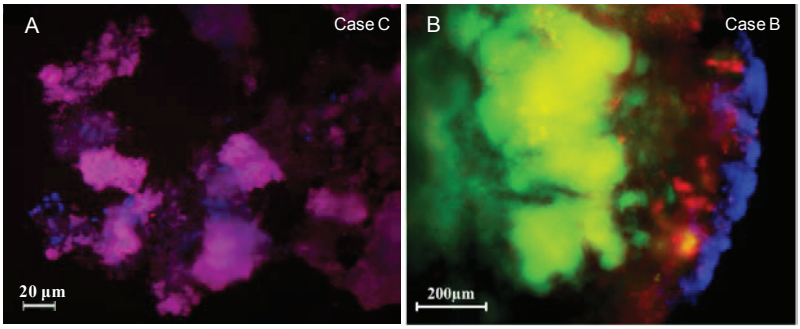

Figure 2. 3 Bacterial population shown by FISH analysis of A) flocculent biomass in the SBR for Case C: anammox species Candidatus 'Brocadia fulgida' (pink), all bacteria (blue); and B) sliced granule of the bubble column SBR for Case B: anammox species Candidatus 'Brocadia fulgida' (green), PAOs (red) and AOB (blue). The oligonucleotide probes used are listed in Table 2. 2.

For Case A, the biomass was taken from a full-scale anammox reactor in which the COD/N ratio was relatively low, hindering the development of heterotrophic bacteria. The SBR of Case C was not exposed continuously to acetate (see Figure 2. 2), which did not favoured the presence of heterotrophs.

The granular sludge for the batch tests of Case B was harvested from a lab reactor exposed to acetate. The FISH determination of this biomass revealed anammox bacteria and PAOs in the inner core of the granules, in addition to AOB in the outer zone (Figure 2. 3B). The reason why PAOs could have developed in the SBR of Case B over time is because little nitrate accumulated in the reactor, since the nitrate produced within the cycle of the reactor was recycled and removed through DNRA or denitrification by adding acetate (Winkler et al. 2012a). Thus, anaerobic conditions (no electron acceptor present) were established and the growth of PAOs promoted, which under anaerobic conditions can accumulate acetate internally as poly-β-hydroxybutyrate (PHB) and under aerobic or anoxic conditions can oxidize the internally stored PHB and use the available energy for growth .

2.3.2 Anammox conversion and organotrophic nitrate reduction

The anammox activity was assessed in the absence (section 2.3.2*a*) and presence of acetate (section 2.3.2*b*) in the medium, as well as the nitrate and acetate consumption rates in the cases with acetate addition. An overview of the batch tests performed to carry out these determinations are presented in Figure 2. 4 (Case A) and Figure 2. 5 (Case B).

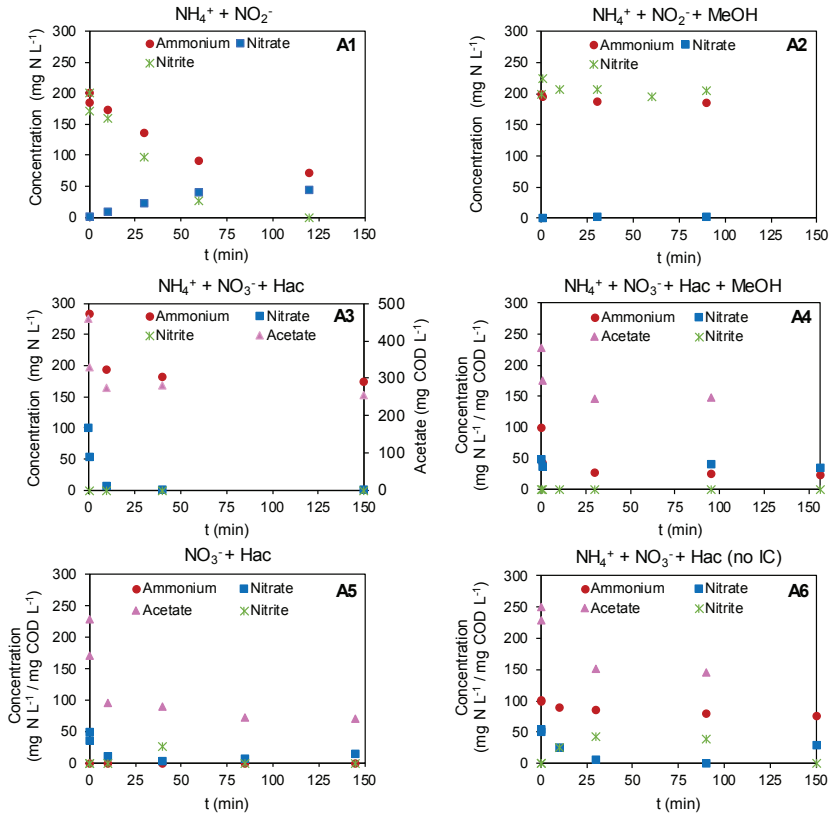

Figure 2. 4 Batch tests performed with anammox biomass from a full-scale reactor (Case A).

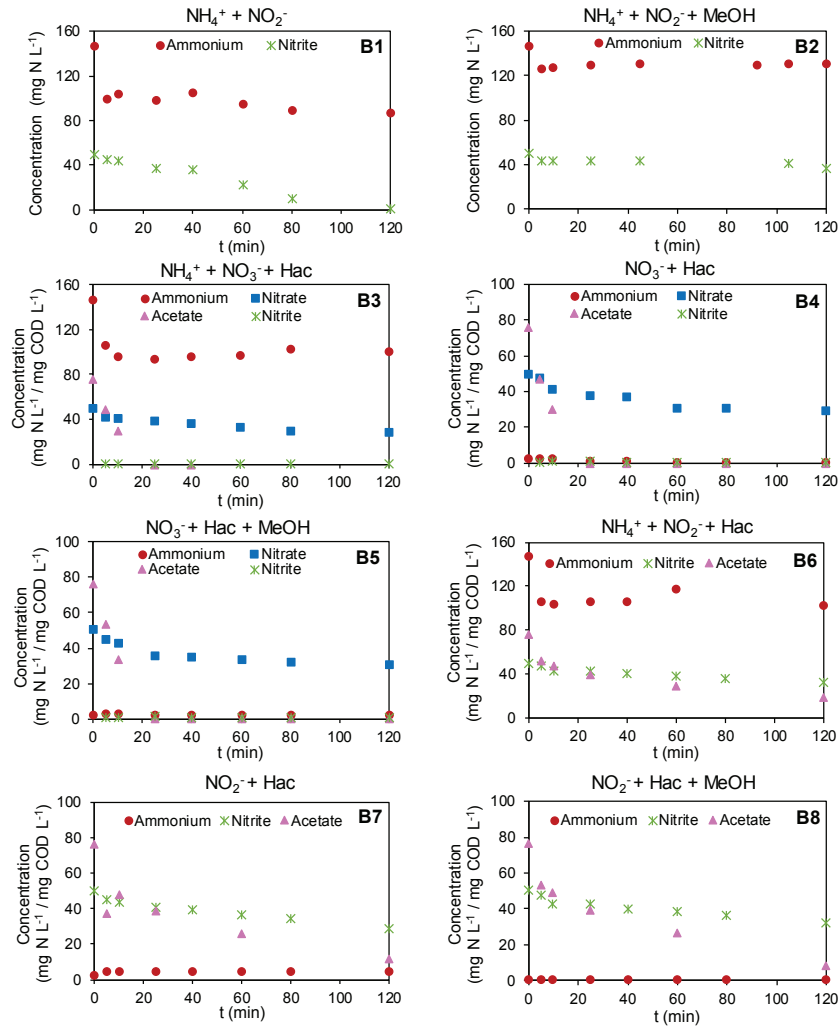

Figure 2. 5 Batch tests with biomass from the SBR with anammox and heterotrophic bacteria (Case B).

a) Anammox activity in the absence of acetate

The anammox activity without organic feeding (acetate) was assessed during Case A (batch tests A.1 and A.2 in Figure 2. 4 and Table 2. 1), Case B (batch tests B.1 and B.2 in Figure 2. 5 and Table 2. 1), and Case C during the 'Anammox regime' (Feed C.1 in Table 2. 1). The results of the specific ammonium (r_{NH4}) and nitrite (r_{NO2}) specific consumption rates are shown in Figure 2. 6. Since r_{NH4} and r_{NO2} are expressed per g VSS (total biomass), they

were higher for the reactors containing nearly only anammox biomass (Cases A and C) than for the reactor containing also PAOs (Case B) (Figure 2. 6).

The addition of MeOH inhibited the anammox activity (r_{NH4}) by 94.0% and 89.3% in Cases A and B respectively (Figure 2. 6A and B). The inhibition observed was not complete as previously reported (Guven et al. 2005), likely due to diffusion limitations or a protective environment inside the granules.

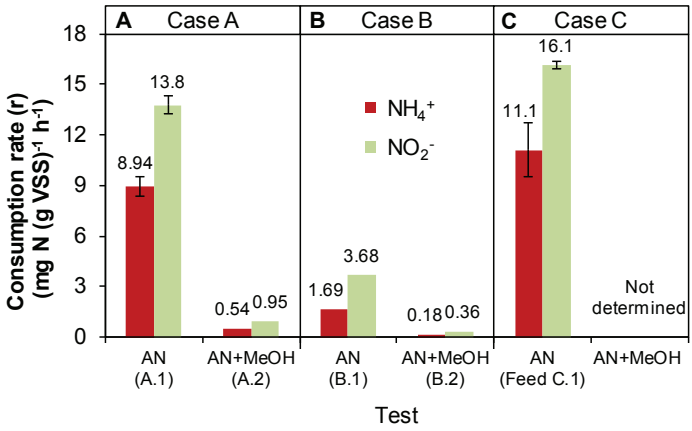

Figure 2. 6 Anammox activities during the A) batch tests of Case A, with biomass from a full-scale anammox granular sludge reactor (A.1, A.2 and A.3); B) batch tests of Case B, with biomass from a SBR with high presence of heterotrophic bacteria (B.1 and B.2), and C) operation of the SBR of Case C under the 'Anammox regime' (Feed C.1); when having a media composition with (AN+MeOH) and without (AN) methanol. The corresponding concentrations of each test are listed in Table 2. 1.

b) Anammox and organotrophic nitrate reduction activities in the presence of acetate

The anammox activity (r_{NH4}) was decreased by 42.1% in Case A and 41.9% in Case B (Figure 2. 7.1) in the presence of acetate, nitrate and ammonium, if compared to the case with ammonium and nitrite, but not acetate (Figure 2. 6). When nitrite is not added, this needs to be formed from the reduction of nitrate, which becomes the limiting reaction, slower than the conventional anammox reaction. Note that the nitrate reduction rates (r_{NO3}) for both Cases A and B in Figure 2. 7.1 and 7.2 are slower than the nitrite reduction (r_{NO2}) during regular anammox reaction in Figure 2. 6A and B.

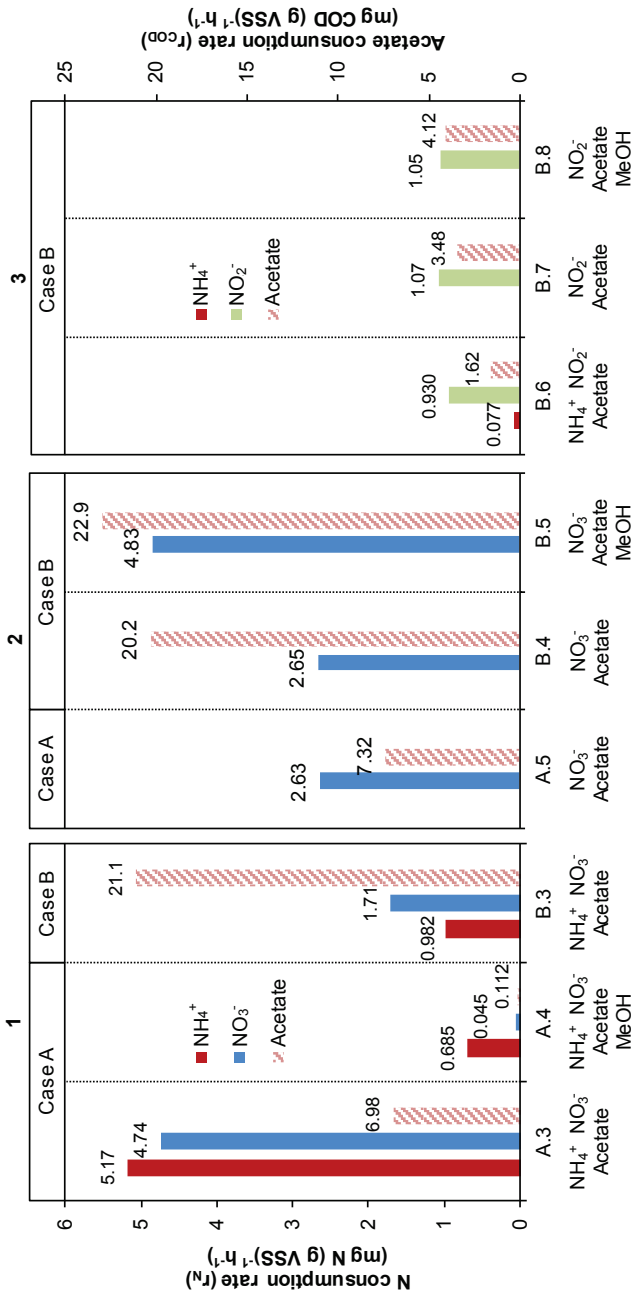

Figure 2. 7 Influence of acetate on the ammonium, nitrate, nitrite and acetate consumption rates (r_{NH4}, r_{NO3}, r_{NO2} and r_{COD}). Results during the batch tests with anammox biomass from the full-scale granular sludge reactor of Case A (A.3 to A.5, Table 2. 1) and from the SBR containing anammox and heterotrophic bacteria of Case B (B.3 to B.8, Table 2. 1) under different feeding conditions.

In order to clarify which communities (anammox or heterotrophic bacteria) reduced nitrate and oxidized acetate (Figure 2. 7), methanol was supplied in the medium together with ammonium, nitrate and acetate for Case A (batch test A.4, Figure 2. 7.1). The addition of methanol inhibited the nitrate reduction and acetate oxidation by 99.0% and 98.4%, respectively, if compared to the test without methanol addition (see batch tests A.3 and A.4 in Figure 2. 7.1). Since earlier studies have shown that heterotrophs are not inhibited at concentrations used in this study (576 g MeOH m^{-3} for Case A, Table 2. 1) (Jensen et al. 2007), it can be concluded that anammox bacteria were the major responsible microorganisms for the consumption of nitrate and acetate in Case A. The anammox biomass used for the experiments in Case A was not adapted to organic matter. This biomass was taken from a full-scale anammox reactor preceded by a SHARON reactor in which some organic carbon that may be present would be oxidized. Despite this fact, in the present study it was shown that the anammox species *Candidatus* 'Brocadia fulgida' can perform DNRA along with anaerobic ammonium oxidation at a high rate. The nitrate reduction rate by anammox bacteria was 1.9 slower than the conventional anammox activity (ammonium oxidation rate in the test with ammonium and nitrite only), which is significantly high compared to previous studies (nitrate reduction 21 times slower than regular anammox activity in the tests from Kartal et al. (2007b) and 10 times slower in Kartal et al. (2007a)).

In Case B, which experiments were conducted with biomass from a lab reactor with relatively high presence of PAOs and/or other heterotrophic populations (Figure 2. 3B), it could not be concluded in what extent heterotrophic and/or anammox bacteria contributed to the reduction of nitrate and oxidation of acetate when supplying ammonium, nitrate and acetate in the medium (B.3 in Figure 2. 7.1). A higher acetate consumption rate (r_{COD}) was found during the batch tests with biomass from the lab scale reactor containing PAOs (Case B, 21.1 mg COD $(g\ VSS)^{-1}\ h^{-1}$) than in the batch tests with anammox biomass from the full-scale system (Case A, 6.99 mg COD $(g\ VSS)^{-1}\ h^{-1}$) . This higher activity can be attributed to PAOs, which are known to have a high acetate uptake rate due to their capacity storing acetate as PHB. Note that during the tests with

nitrate and acetate (B3, B4 and B5 in Figure 2. 7) uptake of phosphorus was observed (Table 2. 3), confirming PAO activity.

Table 2. 3 Phosphorus measurements at the end of the batch tests with granular sludge containing anammox and heterotrophic (PAOs) bacteria (Case B).

Batch tests Case B		P release (mg PO_4^{2-} L^{-1})
B.1	**NH_4^+ + NO_2^-**	not measured
B.2	**NH_4^+ + NO_2^- + MeOH**	5.5
B.3	**NH_4^+ + NO_3^- + Acetate**	0.2
B.4	**NO_3^- + Acetate**	0.0
B.5	**NO_3^- + Acetate + MeOH**	0.0
B.6	**NH_4^+ + NO_2^- + Acetate**	22.8
B.7	**NO_2^- + Acetate**	22.4
B.8	**NO_2^- + Acetate + MeOH**	22.6

2.3.3 What triggers DNRA by anammox bacteria over heterotrophic DNRA and heterotrophic denitrification?

The present study aims to assess when denitrification and DNRA occur by either heterotrophs (PAOs and/or ordinary) or anammox bacteria. Factors influencing DNRA by anammox bacteria were studied experimentally (Figure 2. 6 and Figure 2. 7) and by thermodynamic calculations (Table 2. 4) to distinguish the carbon and nitrogen transformations among heterotrophs and anammox bacteria.

a) Effect of ammonium in the medium on the organotrophic nitrate reduction by anammox bacteria

The effect of ammonium in the medium on the nitrate (r_{NO3}) and COD (r_{COD}) conversion rates was assessed through batch tests with and without the addition of ammonium (Figure 2. 7). For Case A, in which it was concluded earlier (section 2.3.2*b*) that anammox bacteria were the dominating culture and responsible for the nitrate and acetate consumption, the reduction of nitrate was faster in the presence of ammonium (tests A.3 vs. A5, Figure 2. 7) and no accumulation of nitrite was observed (see A3 in Figure 2. 4). Therefore, nitrite must have been supplied to perform the regular anammox reaction through the anammox mediated DNRA pathway in which nitrite is formed as

intermediate (conversions by anammox bacteria in Figure 2. 1C), whereas the absence of ammonium (and nitrite) (A.5 in Figure 2. 7) led to the accumulation of nitrite in the beginning of the test (see A5 in Figure 2. 4), which can be explained by the fact that the reduction of nitrate to nitrite by anammox bacteria is faster than the conversion of nitrite to ammonium (Kartal et al. 2007a)). Within this test without ammonium addition (A.5) no ammonium was accumulated, suggesting that anammox bacteria immediately metabolized the ammonium obtained through full DNRA for their regular anammox reaction (conversion by anammox bacteria in Figure 2. 1D).

Anammox bacteria are, by using DNRA, able to expand their growth niche from pure autotrophic with ammonium and nitrite present to ecological niches with only nitrate and acetate present. The DNRA pathway has not been shown to deliver net energy to the organisms but supplies them with their catabolic substrates. Depending on the conditions, only nitrite is produced (via partial DNRA) which is then reduced with ammonium present in the environment (Figure 2. 1C) or DNRA produces both nitrite an ammonium (Figure 2. 1D).

Interestingly, the presence of ammonium enhances the organotrophic reduction rate of nitrate by anammox bacteria since the slow formation of ammonium through DNRA is avoided and no nitrite is accumulated (Case A). This is in line with other studies in which the DNRA reactions by anammox bacteria were predominant over heterotrophic transformations in the presence of ammonium (Guven et al. 2005; Kartal et al. 2007a; Kartal et al. 2007b; Winkler et al. 2012a; Winkler et al. 2012b).

b) Effect of substrate limitation - energy perspective

The energy obtained per substrate consumed, N_2 formed, and the electrons being transferred were calculated for different microbial pathways (see Appendix A2.2 for description of the calculation) since it is one of the main driving forces explaining microbial competition (Gonzalez-Cabaleiro et al. 2015) (Table 2. 4).

Table 2. 4 Catabolic reaction stoichiometry, Gibbs free energy per substrate, N_2 produced and electron transferred, and stoichiometric ratio COD/NO_3^- for different processes.

	Biological process	Reaction	kJ $(e^-eq)^{-1}$	kJ (mol NO_3^- or $NO_2^-)^{-1}$	kJ (mol acetate)$^{-1}$	kJ (mol $N_2)^{-1}$	Stoichiometric COD/NO_3^- (g COD (g N)$^{-1}$)
1	**Denitrification**	$\mathbf{8NO_3^-}$ + $5CH_3COO^-$ + $8H^+$ → $5CO_2$ + $\mathbf{4N_2}$ + $5HCO_3^-$ + $9H_2O$	-99.60	-498.0	-796.8	-996.0	2.86
2	**DNRA**	$\mathbf{NO_3^-}$ + CH_3COO^- + $2H^+$ → CO_2 + $\mathbf{NH_4^+}$ + HCO_3^-	-62.51	-500.1	-500.1		4.57
3	**Partial DNRA/ Denitrification[1]**	$\mathbf{4NO_3^-}$ + CH_3COO^- → CO_2 + $\mathbf{4NO_2^-}$ + HCO_3^- + H_2O	-69.05	-138.1	-552.4		1.14
4	**Nitrite reduced via Anammox**	$\mathbf{NO_2^-}$ + $\mathbf{NH_4^+}$ → $\mathbf{N_2}$ + $2H_2O$	-119.3	-357.8		-357.8	
5	**Nitrite reduced via DNRA**	$\mathbf{4NO_2^-}$ + $3CH_3COO^-$ + $8H^+$ + H_2O → $3CO_2$ + $3HCO_3^-$ + $\mathbf{4NH_4^+}$	-60.33	-362.0	-482.6		3.43
6	**Nitrite reduced via Denitrification**	$\mathbf{8NO_2^-}$ + $3CH_3COO^-$ + $8H^+$ → $3CO_2$ + $3HCO_3^-$ + $\mathbf{4N_2}$ + $7H_2O$	-119.96	-359.9	-959.7	-719.8	1.71
7	**Partial DNRA + Anammox[2]**	$\mathbf{4NO_3^-}$ + CH_3COO^- + $\mathbf{4NH_4^+}$ → CO_2 + HCO_3^- + $\mathbf{4N_2}$ + $9H_2O$	-99.18	-495.9	-1984	-495.9	1.14
8	**Full DNRA + Anammox[3]**	$\mathbf{8NO_3^-}$ + $5CH_3COO^-$ + $8H^+$ → $5CO_2$ + $5HCO_3^-$ + $\mathbf{4N_2}$ + $9H_2O$	-99.60	-498.0	-796.8	-996.0	2.86

* Partial DNRA = partial denitrification, i.e. reduction of nitrate to nitrite only
** All the NH_4^+ comes from the environment, i.e. only the first step of the DNRA process occurs (NO_3^- → NO_2^-) (Figure 2. 1C)
*** All NH_4^+ comes from the DNRA process, i.e. all NO_3^- is converted to NO_2^- and half of the NO_2^- is further converted to NH_4^+ (Figure 2. 1D)

According to the energy per electron transferred for the heterotrophic transformations (Table 2. 4), a higher theoretical yield is obtained during the denitrification of nitrite (reaction 6, -120 kJ e^--eq^{-1}), then it is the case for the denitrification of nitrate (reaction 1, -100 kJ e^--eq^{-1}) whereas the DNRA is the least favoured process for heterotrophic transformations (reaction 2, -62.5 kJ e^--eq^{-1}). Therefore, under none substrate limiting conditions, heterotrophs would be more favoured if nitrite is available in the medium. With respect to substrate competition, van den Berg et al. (2015) selected heterotrophic communities performing DNRA over denitrification when limiting the nitrate in the system (high COD/N ratio). However, from a thermodynamic point of view, the limitation of nitrate (such as applied in van der Bergs research) cannot be used to explain the dominance of the heterotrophic DNRA pathway because a similar energy is harvested per mole of nitrate during denitrification (reaction 1) and DNRA (reaction 2, Table 2. 4). Instead, the limitation of acetate (low COD/N ratio) would promote the heterotrophic denitrification (-797 kJ mol-$acetate^{-1}$) over the heterotrophic DNRA (-500 kJ mol-$acetate^{-1}$).

Regarding the conversions involving anammox bacteria, the theoretical Gibbs free energy obtained per N_2-mol produced is higher for the partial DNRA-anammox (reaction 7, Figure 2. 1C) and full DNRA-anammox (reaction 8, Figure 2. 1D) than the energy obtained if only doing conventional anaerobic ammonium oxidation (reaction 4). Nevertheless, whether or not anammox bacteria can use this extra energy when performing the DNRA pathway and in which way is not yet clearly understood. Current literature suggests that anammox bacteria do not use the organic carbon for cell assimilation and therefore energy usage from this pathway remains unsolved (Kartal et al. 2013). Following this thermodynamic approach, the limitation of acetate could be the key to promote the partial DNRA-anammox (reaction 7, -1984 kJ mol-$acetate^{-1}$) over both heterotrophic DNRA (reaction 2) and heterotrophic denitrification (reaction 1), as well as the full DNRA-anammox (reaction 8). This can be explained because more energy is generated when supplying externally the ammonium (partial DNRA-anammox, Figure 2. 1C) than when forming the ammonium through DNRA and then converting it during the

regular anammox reaction (full DNRA-anammox, Figure 2. 1D). Also, the partial DNRA-anammox is the only pathway in which anammox bacteria could outcompete heterotrophic denitrification from a thermodynamic perspective when limiting the carbon source (more energy is produced, 1984 kJ acetate-mol^{-1} during partial DNRA-anammox vs. 797 during heterotrophic denitrification). Although the energy usage of the anammox driven DNRA pathway is not unravelled yet, experimental results have shown that heterotrophic transformations can be outcompeted by anammox bacteria performing DNRA at low COD/N ratios (Winkler et al. 2012b). Overall it must be noted that anammox can generate ammonium and/or nitrite from the DNRA pathway and hence support their autotrophic pathway under conditions in which they are lacking their electron acceptor or donor (or both). This therefore yields in an energy gain irrespective from the DNRA pathway.

c) Effect of inorganic carbon

The DNRA reaction yields inorganic carbon (reaction 2 in Table 2. 4), while the anammox reaction uses inorganic carbon for biomass growth. The effect of inorganic carbon on the combined partial DNRA-anammox transformation by anammox bacteria was investigated with an anammox SBR, which was operated alternating a feeding with ammonium, nitrate and acetate, and with ammonium and nitrite, without any addition of inorganic carbon (description of reactor operation in section 2.2.3).

The r_{NH4}, r_{NO3} and r_{COD} were studied under two different COD/NO_3^--N ratios in the influent (1.1 and 0.6 g COD (g N)$^{-1}$, Figure 2. 8A and B, respectively). For the feeding ratio COD/NO_3^--N = 1.1, the consumption rate ratios obtained (r_{COD}/r_{NO3} = 1.61 and 1.19 in Figure 2.8A) were relatively similar to the stoichiometric ratios COD/NO_3^--N corresponding with partial DNRA-anammox (1.14 in Table 2. 4, reaction 7), if compared to the stoichiometric COD/NO_3^--N ratios of the other transformations in Table 2. 4. This, together with the fact that no significant biomass growth was observed (average biomass concentration during the operation of the SBR = 1.14 ±0.02 g VSS L^{-1}), suggests that partial DNRA-anammox by anammox bacteria took place

during the feeding ratio COD/NO_3^--N of 1.1 and not heterotrophic conversions. The inorganic carbon produced during the partial DNRA by anammox bacteria supported the regular autotrophic anammox reaction.

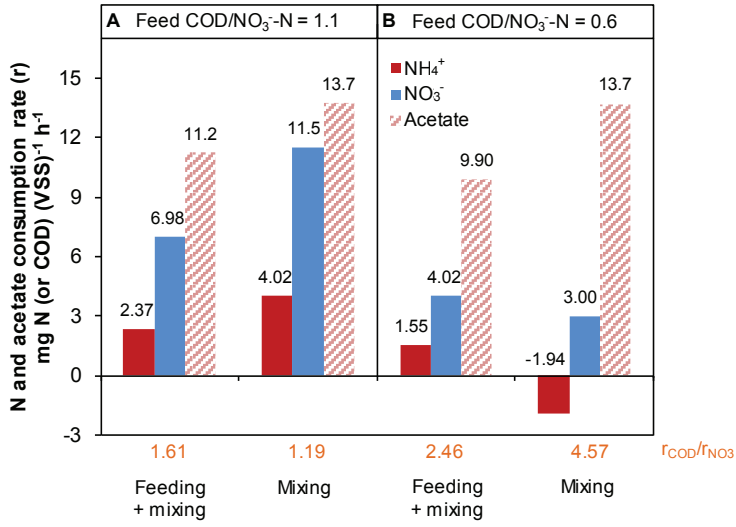

Figure 2. 8 Effect of inorganic carbon absence on the ammonium, nitrate and acetate consumption rates (r_{NH4}, r_{NO3} and r_{COD}) for the 'Regime under semi-continuous exposure to acetate' in Case C under two COD/NO_3^--N feeding ratios.

For the case with the feeding ratio COD/NO_3^--N of 0.6, the r_{NH4} got a negative value during the mixing step, indicating formation of ammonium through DNRA, and the r_{NO3} decreased by 62.1%. With a lower feeding ratio, the inorganic carbon formation through partial DNRA was lower since less COD was added and lower oxidation of acetate by DNRA occurred, thus limiting the anammox activity.

Overall, the inorganic carbon needs to be sufficient to obtain the combined partial DNRA-anammox by anammox bacteria. This inorganic carbon could be produced during the DNRA pathway to support the autotrophic anammox reaction. While the limitation of organic carbon (low COD/N ratio in the influent) favours the partial DNRA-anammox conversion from a thermodynamic perspective (section 2.3.3*b*), this consideration becomes less relevant when the bacteria are impeded to grow (limitation of inorganic carbon).

2.3.4 Anammox conversion in the presence of heterotrophic bacteria

Batch tests were performed with nitrite, nitrate, and acetate for Case B (high presence of heterotrophic bacteria, PAOs) (Figure 2. 7) to assess the competitiveness of the anammox pathway in the presence of heterotrophic bacteria and organic carbon compounds (acetate). Since the addition of methanol did not inhibit the nitrate/nitrite and acetate consumption rates in the absence of ammonium (B.4 vs. B.5 and B.7 vs. B.8 in Figure 2. 7), it is clear that heterotrophic transformations were the dominant reactions (Jensen et al. 2007) consuming nitrate/nitrite and acetate during these batch tests. When ammonium was added (together with nitrate/nitrite, and acetate) (B.3 and B.6 in Figure 2. 7), it was found that anammox bacteria oxidized ammonium in the presence of nitrate (r_{NH4} = 0.982 mg N $(gVSS)^{-1}$ h^{-1}), but negligible consumption was found in the experiments with nitrite (r_{NH4} = 0.077 mg N (g VSS)$^{-1}$ h^{-1}). Anammox bacteria were competitive for the nitrite formed from the reduction of nitrate (most likely by heterotrophs) when having acetate present and heterotrophic bacteria. This could be explained by a limitation on the heterotrophic denitrification since the acetate was completely consumed around minute 20 after starting the batch tests with nitrate (see B3, B4 and B5 in Figure 2. 5). Although PAOs could use some PHB stored to denitrify nitrite, anammox conversion was competitive. The addition of nitrite likely inhibited the anammox conversion, since nitrite was available during the entire batch test (B6 in Figure 2. 5), showing also certain inhibition on the PAO activity or low capacity of PAOs to utilize nitrite, since the r_{NO2} were significantly lower than the r_{NO3} in the experiments of Case B (Figure 2. 7).

The organic carbon limitation (acetate completely consumed) together with the presence of nitrate can benefit the anammox reaction, since the heterotrophic denitrification yields nitrite that can be used along with ammonium by anammox bacteria (partial denitrification-anammox, Figure 2. 1B). This finding are in line with the results obtained in the modelling study of Mozumder et al. (2014), in which the authors pointed out that low

concentrations of COD could benefit the anammox reaction by the heterotrophic reduction of nitrate to nitrite.

2.3.5 Partial DNRA-anammox by anammox bacteria for more sustainable municipal wastewater treatment

The main bottleneck to implement anammox technology in the mainstream of the WWTP is an effective supply of nitrite to the anammox reaction (Ma et al. 2016), which is troublesome due to the hard suppression of NOB at low temperatures (Lotti et al. 2015). If the DNRA pathway of anammox bacteria could be promoted in the mainstream, the suppression of NOB would not be so crucial anymore since nitrate would be reduced by anammox bacteria with some organic carbon, while keeping sludge production low given that anammox bacteria do not grow on organic substrates (Kartal et al. 2007b; Winkler et al. 2012a). The feasibility of the partial DNRA-anammox conversion (reaction 7 in Table 2. 4 and Figure 2. 1C) by anammox bacteria was demonstrated in this study (Figure 2. 7) and by previous researches (Guven et al. 2005; Kartal et al. 2007a; Winkler et al. 2012a; Winkler et al. 2012b).

In order to decrease the nitrate accumulation caused by NOB (or anammox itself) in the mainstream, we suggest to make use of the partial DNRA-anammox process by anammox bacteria, by implementing it for enhanced nitrogen removal according to the scheme shown in Figure 2. 9. In the suggested approach the organic matter from the wastewater is removed in a high rate activated sludge (HRAS) system and captured in biomass, which is used to maximize the energy recovery in the digester (Jetten et al. 1997). Since the HRAS systems are operated at low hydraulic retention time, nitrification is prevented and the effluent is high in ammonium. The effluent from the HRAS unit is directed to the nitrogen removal system, which consists of partial nitrification combined with partial DNRA-anammox by anammox bacteria (Figure 2. 1C). This system not only would remove the nitrate generated by NOB but also the nitrate produced during the anammox conversion itself, thus increasing the effluent quality. In order to promote the partial DNRA pathway by anammox bacteria, volatile fatty acids (VFA) need

to be added to the system. These VFA could be generated onsite in a small fermentation unit receiving sludge from the HRAS reactor. The production of organic acids is in general gaining increasing interest as an alternative to conventional biogas production in anaerobic digesters (Cagnetta et al. ; Khiewwijit et al. 2015; Kleerebezem et al. 2015). The external supply of VFA would cost some money but if compared to conventional nitrification-denitrification systems, up to 50% energy savings in aeration would be obtained by applying partial DNRA-anammox, since only half of the ammonium would be oxidized to nitrate. Assuming an average influent soluble COD concentration in the plant of 270 g COD m^{-3} (Henze et al. 2008), influent ammonium concentration of 45 g N m^{-3} and ammonium concentration of 1000 g N m^{-3} leaving the fermenter and/or possible combined digester, the COD needed to carry out the partial DNRA would be around 21% of the incoming soluble COD in the plant. A detailed calculation based on nitrogen and COD mass balances is presented in the Appendix A2.3.

Regarding the practical implementation of the nitrogen removal system in Figure 2. 6, two configurations are suggested.

a) One-unit configuration aerobic/anoxic

Half of the ammonium is converted to nitrate by NOB during the aerobic step. Following, partial DNRA-anammox by anammox bacteria takes place with VFA addition during the anoxic period, while the oxidation of nitrite by NOB is avoided (aerobic organisms). Actually, under anoxic conditions, NOB could use their mixotrophic capacity to reduce nitrate (Freitag et al. 1987), which in that case would benefit the performance of the system. In a lab-scale which followed a similar approach, Winkler et al. (2012a) proved the capability of anammox bacteria to perform DNRA and to remove the nitrate produced during partial nitritation-anammox conversion.

b) Two-unit configuration

In this configuration the aerobic and anoxic phases are carried out in separated reactors. Half of the ammonium is oxidized to nitrate in the aerobic unit and the other half is removed in a second anoxic anammox reactor fed

with VFA, thus performing the partial DNRA together with the anammox reaction. One potential limitation for this configuration is the insufficient availability of alkalinity in the wastewater to carry out the nitrification. Partial recirculation from the second unit to the first one could avoid alkalinity and pH problems since inorganic carbon is formed during the partial DNRA pathway (reaction 7 in Table 2. 4).

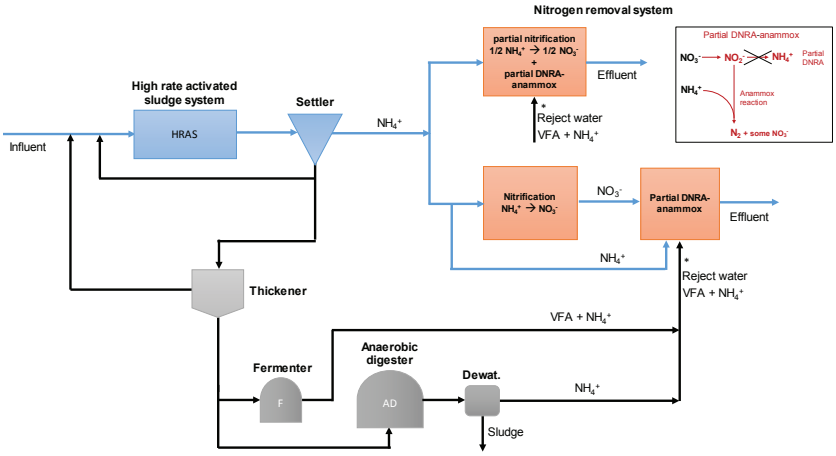

Figure 2. 9 Suggested scheme for more sustainable municipal wastewater treatment with nitrification + partial DNRA-anammox by anammox bacteria as nitrogen removal system in both proposed one and two unit configurations.

In these two configurations the first aerobic step could still aim for partial nitritation and the accumulation of nitrite. However, it was assumed that half of the ammonium is converted to nitrate for simplicity and since partial nitritation was not achieved so far in the mainstream (Lotti et al. 2015). Also, stable operation conditions are a critical point during mainstream wastewater treatment, which are more difficult to obtain when aiming at nitritation ($NH_4^+ \rightarrow NO_2^-$) instead of nitrification ($NH_4^+ \rightarrow NO_3^-$).

The use of granular sludge technology is recommended to avoid the implementation of settlers for the biomass separation, leading to compact and high rate process units.

Overall, all aspects discussed in section 2.3.3 and 2.3.4 would facilitate the success of the partial DNRA-anammox process. The presence of ammonium in

the medium would favour the out-competition of heterotrophs, as shown in this study (Figure 2. 7) and by others (Kartal et al. 2007b; Kartal et al. 2008). Also, when ammonium is available in the medium, the conversion of nitrite to ammonium by anammox bacteria is prevented (full-DNRA) since this conversion is slower than the nitrate reduction to nitrite (Figure 2. 7), thus promoting partial DNRA-anammox (conversion in Figure 2. 1C instead of D). If the COD is limited (low COD/N influent ratio), heterotrophic denitrification and heterotrophic and anammox full DNRA are disfavoured over partial DNRA-anammox from a thermodynamic perspective, since the later process produces more Gibbs free energy per mole of organic molecule consumed (Table 2. 4). The presence of nitrate instead of nitrite could make anammox bacteria competitive for the nitrite formed during partial DNRA or partial denitrification (Figure 2. 7 and Mozumder et al. (2014)). However, as shown in Figure 2. 8, the insufficient availability of inorganic carbon for anammox bacteria to grow could impede the combined partial DNRA-anammox process. Furthermore, *Candidatus* 'Brocadia fulgida', a common species in full-scale WWTPs and found in this study (Figure 2. 3), has proven to be a good organism performing partial DNRA-anammox, without previous adaptation to VFA (Figure 2. 7.1).

This study is the first one suggesting the exploitation of the organotrophic nitrate reduction capacity of anammox bacteria to facilitate the implementation of the regular anammox conversion in the main line of the wastewater treatment plant. However, further research is needed to proof the stability of the process long-term.

2.4 Conclusions

The competition among heterotrophs and anammox bacteria for organic carbon and nitrate was studied to gain understanding on the DNRA capacity of anammox bacteria. The species *Candidatus* 'brocadia fulgida' showed high DNRA performance even when the culture was not previously adapted to organic carbon (acetate). Gibbs free energy calculations showed that limiting organic carbon (low COD/N influent ratio) favoured the partial DNRA

($NO_3^- \rightarrow NO_2^-$)-anammox conversion over the heterotrophic DNRA and heterotrophic denitrification. Also, the presence of ammonium and sufficient inorganic carbon enhanced the process. Overall, the combined partial DNRA-anammox could be applied for sustainable nitrogen removal from municipal wastewater, but still further research needs to be devoted to assess the long-term stability of this novel process.

Acknowledgements

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Appendix

A2.1 Mineral medium

The mineral medium used in the batch tests of the Case A and during the set-up of Case C is described in Table A2. 1 and Table A2. 2.

Table A2. 1 Mineral medium (adapted from van de Graaf et al. (1996) and Dapena-Mora et al. (2004)).

Compound	Concentration	Unit
$CaCl_2.H_2O$	300	g m^{-3}
$MgSO_4.7H_2O$	200	g m^{-3}
$FeSO_4$	6.25	g m^{-3}
EDTA	6.25	g m^{-3}
$NaH_2PO_4.2H_2O$	17.6	g P m^{-3}
$Na_2HPO_4.12H_2O$	2.4	g P m^{-3}
Trace element solution (Table A2. 2)	1.25	mL L^{-1}

Table A2. 2 Composition of the trace element solution used.

Compound	g L^{-1}
EDTA	15
$ZnSO_4.7H_2O$	0.43
$CoCl_2.6H_2O$	0.24
$MnCl_2.4H_2O$	0.99
$CuSO_4.5H_2O$	0.25
$Na_2MoO_4.2H_2O$	0.22
$NiCl_2.6H_2O$	0.19
$NaSeO_4.10H_2O$	0.21
H_3BO_3	0.014

A2.2 Reaction stoichiometry and Gibbs free energy calculation

The Gibbs free energy and stoichiometry of different reactions were calculated based on the half reactions of Table A2. 3 and are described in Table A2. 4.

Table A2. 3 Half reactions described in Rittmann and McCarty (2001).

	Half reactions	$\Delta G^{o'}$ kJ $(e^-eq)^{-1}$
A	$1/8\ NO_3^- + 5/4\ H^+ + e^- = 1/8\ NH_4^+ + 3/8\ H_2O$	-35.11
B	$1/6\ NO_2^- + 4/3\ H^+ + e^- = 1/6\ NH_4^+ + 1/3\ H_2O$	-32.93
C	$1/2\ NO_3^- + H^+ + e^- = 1/2\ NO_2^- + 1/2\ H_2O$	-41.65
D	$1/5\ NO_3^- + 6/5\ H^+ + e^- = 1/10\ N_2 + 3/5\ H_2O$	-72.20
E	$1/3\ NO_2^- + 4/3\ H^+ + e^- = 1/6\ N_2 + 2/3\ H_2O$	-92.56
F	$1/6\ N_2 + 4/3\ H^+ + e^- = 1/3\ NH_4^+$	26.70
G	$1/4\ O_2 + H^+ + e^- = 1/2\ H_2O$	-78.72
H	$1/8\ CO_2 + 1/8\ HCO_3^- + H^+ + e^- = 1/8\ CH_3COO^- + 3/8\ H_2O$	27.40
I	$1/2\ HCO_3^- + H^+ + e^- = 1/2\ HCOO^- + 1/2\ H_2O$	39.19
J	$1/4\ CO_2 + H^+ + e^- = 1/24\ C_6H_{12}O_6 + 1/4\ H_2O$	41.35

Table A2. 4 Calculation of the Gibbs free energy and stoichiometry of different reactions.

Biological process	Reaction	e- transf.	Relation with half reactions	kJ $(e^-eq)^{-1}$	kJ (mol NO_3^- or NO_2^-)$^{-1}$	kJ (mol acetate)$^{-1}$	kJ (mol N_2)$^{-1}$
Denitrification	$\mathbf{8NO_3^-}$ + $5CH_3COO^-$ + $8H^+$ → $5CO_2$ + $\mathbf{4N_2}$ + $5HCO_3^-$ + $9H_2O$	40	40(D-H)	-99.60	-498.0	-796.8	-996.0
DNRA	$\mathbf{NO_3^-}$ + CH_3COO^- + $2H^+$ → CO_2 + $\mathbf{NH_4^+}$ + HCO_3^-	8	8(A-H)	-62.51	-500.1	-500.1	
Partial DNRA/Denitrification[1]	$\mathbf{4NO_3^-}$ + CH_3COO^- → CO_2 + $\mathbf{4NO_2^-}$ + HCO_3^- + H_2O	8	8(C-H)	-69.05	-138.1	-552.4	
Nitrite reduced via Anammox	$\mathbf{NO_2^-}$ + $\mathbf{NH_4^+}$ → $\mathbf{N_2}$ + $2H_2O$	3	3(E-F)	-119.3	-357.8		-357.8
Nitrite reduced via DNRA	$\mathbf{4NO_2^-}$ + $3CH_3COO^-$ + $8H^+$ + H_2O → $3CO_2$ + $3HCO_3^-$ + $\mathbf{4NH_4^+}$	24	24(B-H)	-60.33	-362.0	-482.6	
Nitrite reduced via Denitrification	$\mathbf{8NO_2^-}$ + $3CH_3COO^-$ + $8H^+$ → $3CO_2$ + $3HCO_3^-$ + $\mathbf{4N_2}$ + $7H_2O$	24	24(E-H)	-119.96	-359.9	-959.7	-719.8
Partial DNRA + Anammox[2]	$\mathbf{4NO_3^-}$ + CH_3COO^- + $\mathbf{4NH_4^+}$ → CO_2 + HCO_3^- + $\mathbf{4N_2}$ + $9H_2O$	20	8(C-H) + 12(E-F)	-99.18	-495.9	-1984	-495.9
Full DNRA + Anammox[3]	$\mathbf{8NO_3^-}$ + $5CH_3COO^-$ + $8H^+$ → $5CO_2$ + $5HCO_3^-$ + $\mathbf{4N_2}$ + $9H_2O$	40	40(D-H)	-99.60	-498.0	-796.8	-996.0

[1] Partial DNRA = partial denitrification, i.e. reduction of nitrate to nitrite only

[2] All the NH_4^+ comes from the environment, i.e. only the first step of the DNRA process occurs (NO_3^- → NO_2^-) (Figure 2. 1C)

[3] All NH_4^+ comes from the DNRA process, i.e. all NO_3^- is converted to NO_2^- and half of the NO_2^- is further converted to NH_4^+ (Figure 2. 1D)

A2.3 Calculation of ammonium and COD concentrations and flows

A simplified calculation based on ammonium and COD mass balances was made to determine the COD that would be necessary to perform the partial DNRA-anammox in the scheme suggested in Figure 2. 9.

For the calculation the two unit configuration for nitrogen removal was taken and all streams were numbered according to Figure A2. 1.

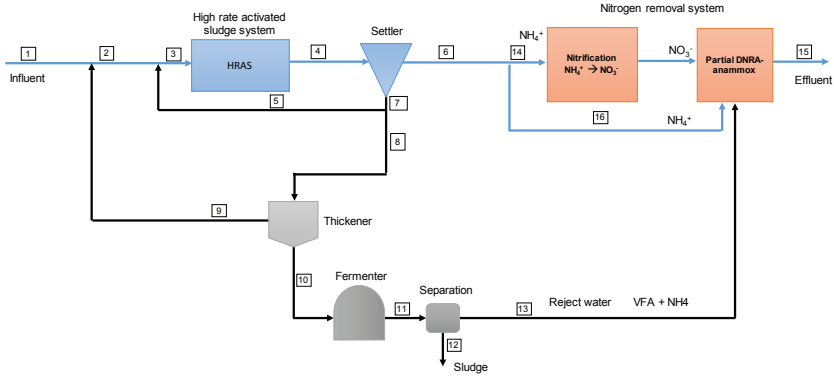


Figure A2. 1 Scheme for the implementation of partial DNRA-anammox considering a fermenter to produce VFA and nitrification (i.e. NOB are not supressed and half of the ammonium is converted to nitrate).

The calculation was based on a fixed amount of reject water generated, 1% of the influent flow rate (ratio of the streams 13/1=0.01). The COD contained in the biomass from the settler goes to a fermenter (not anaerobic digester) to assess the maximum VFA that could be obtained and see if the COD in the wastewater can be sufficient to perform the partial DNRA-anammox transformation.

Assumptions for the calculation:

- Soluble biodegradable COD is considered for the calculation, with an influent value of 270 g COD m^{-3} (medium concentrated municipal wastewater in Henze et al. (2008)). Total COD would be 750 g COD m^{-3}. Note that the biodegradable particulate COD (around 300 g COD m^{-3}) was not considered and this could be hydrolyzed along the treatment, thus increasing the available COD to obtain VFA and/or CH_4 biogas in a

digester. Therefore, the present calculation is relatively conservative by using the value of 270 g COD m^{-3}.

- No difference is made between inorganic nitrogen (ammonium) and organic nitrogen in the influent, using a total value for the influent of 45 g NH_4^+-N m^{-3}. The organic nitrogen would be hydrolyzed in further anaerobic steps. Either way, the ammonium concentration of the reject water from the fermenter was chosen relatively high (1000 g N m^{-3}) to account for the worst case scenario. The calculation of the ammonium leaving the fermenter was not made since this would imply to model and simulate a fermenter, which is not the purpose of this calculation.
- 20% of the soluble COD in the influent is not removed/recovered after the HRAS unit and goes to the nitrogen removal system in the stream 6.
- 30% of the COD that enters the fermenter is not converted to VFA.
- No removal of nitrogen happens in the HRAS system neither in the thickener.
- All COD that enters the thickener is contained in the sludge and goes to the fermenter. Thus, it is assumed that stream 9 does not contain COD.
- Acetate is used to calculate the amount of COD needed for partial DNRA-anammox (Eq. A2.1).

Some ratios among the different streams were fixed to account for the separation efficiency in the units and recirculation, as well as some fractions to account for the COD removal efficiency of the HRAS system and the COD that is not converted to VFA in the fermenter (Table A2. 5).

Table A2. 5 Stream ratios defined based on the steady state simulation of the Benchmark Simulation Model no 2 (Jeppsson et al. 2007). The fraction of COD that is not removed in the HRAS system and goes to the N removal system was set as 20% and the COD contained in the sludge from the fermenter that is not converted to VFA as 30% (stream 12 in Figure A2. 1).

Streams (see Figure A2. 1 for number identification)		Value
Reject water/total inflow	13/1	0.01
Dewatering separation	13/11	0.95
Thickener separation	9/8	0.90
Settler separation	6/4	0.50
Recirculation to HRAS	8/7	0.50
COD to N removal from HRAS	-	0.20
COD in the sludge	-	0.30

The results of the ammonium and COD balances for each stream in Figure A2. 1 are presented in Table A2. 6.

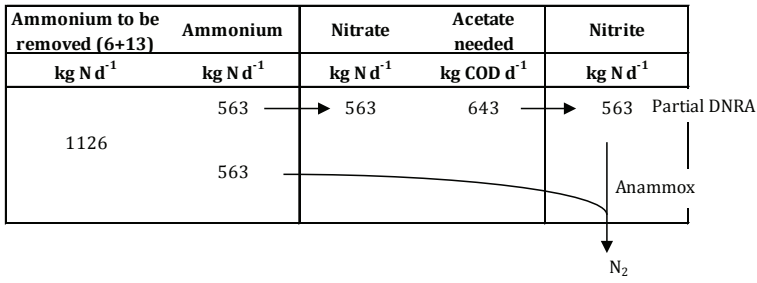
Table A2. 6 Ammonium and COD flows and concentrations obtained after mass balance calculation (see Figure A2. 1 for identification of the streams). Grey shaded values were fixed.

Stream		Flow rate	Ammonium		Soluble biodegradable COD	
		$m^3 d^{-1}$	$g N m^{-3}$	$kg N d^{-1}$	$g COD m^{-3}$	$kg COD d^{-1}$
1	Influent	20648	45.00	929.2	270.0	5575
2	Influent and RW from thickener	22605	45.00	1017	246.6	5575
3	Influent HRAS	24778	45.00	1115	405.0	10035
4	Influent settler	24778	45.00	1115	405.0	10035
5	Recirculation HRAS	2174	45.00	97.81	2052	4460
6	Effluent settler	20431	45.00	919.4	54.57	1115
7	Sludge from settler	4347	45.00	195.6	2052	8920
8	Sludge to thickener	2174	45.00	97.81	2052	4460
9	Reject water from thickener	1956	45.00	88.03	0.000	0.000
10	Sludge to Fermenter	217.4	45.00	9.781	20520	4460
11	Effluent from Fermenter	217.4	1000	217.4	20520	4460
12	Sludge from Fermenter	10.87	1000	10.87	123120	1338
13	Reject water	206.5	1000	206.5	15120	3122
14	Influent N removal system	12510	45.00	562.9	54.57	4.363
15	Effluent	20637	0.000	0.000	120.1	2479
16	NH4 to partial DNRA-anammox	7921	45.00	356.5	54.57	6.890

The total ammonium that needs to be treated comes from the settler (stream 6) and the reject water (stream 13) accounting for 1126 kg N d^{-1}. Assuming that NOB are not supressed, half of the ammonium would be converted to nitrate (1126/2 = 563 kg N d^{-1}) in the nitrification unit (stream 14). According to the partial DNRA transformation (Eq. A2.1), 643 kg COD d^{-1} (as acetate) would be necessary to reduce the nitrate to nitrite, giving 563 kg NO_2^--N d^{-1}. This nitrite can then be combined with the remaining ammonium (563 kg NH_4^+-N d^{-1}) to yield nitrogen gas during the regular anammox reaction. A summary of the calculation if given in Table A2. 7.

Partial DNRA $CH_3COO^- + 4NO_3^- \rightarrow CO_2 + 4NO_2^- + HCO_3^- + H_2O$ [A2.1]

Table A2. 7 Calculation of the nitrogen compounds and acetate (COD) to perform the partial DNRA-anammox conversion.



The soluble biodegradable COD that enters the plant is 5575 kg COD d^{-1} (stream 1). After the calculation and the different assumptions considered, 3122 kg COD d^{-1} (stream 13) could be obtained as VFA in the fermenter. To carry out the partial DNRA, 643 kg COD d^{-1} are necessary (Table A2. 7) which correspond with **20.6%** of the COD recovered as VFA and **11.5%** of the total soluble biodegradable COD in the influent. Therefore, the wastewater contains sufficient COD that could be converted to VFA to carry out the partial DNRA-anammox.

3

Effect of nitrite on the N_2O and NO production on the nitrification of low strength ammonium wastewater

Abstract

The effect of nitrite on N_2O and NO emission was assessed in a nitrifying lab-scale reactor fed with low strength ammonium wastewater. The effect of nitrite pulse additions (5 to 50 mg NO_2^--N L^{-1}) and of step-wisely increasing nitrite concentrations (0 to 150 mg NO_2^--N L^{-1}) was studied. For the pulse addition experiments, N_2O and NO emissions increased upon pulse addition of nitrite, decreasing to the original concentration once nitrite was consumed. The highest peak emissions were detected at nitrite concentrations of 50 mg NO_2^--N L^{-1}. For the step-wisely increasing nitrite tests, N_2O-N emission per NH_4^+-N converted increased linearly from 0.16% to 1.5%. NO emissions were substantially affected at nitrite concentrations higher than 50 mg NO_2^--N L^{-1}, reaching 3.8% NO-N per NH_4^+-N converted at 150 mg NO_2^--N L^{-1}. The results provide one of the first evidences of the combined effect of nitrite on N_2O and NO emissions, showing a stronger effect on NO emissions at high nitrite concentrations.

Chapter redrafted after:

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3.1 Introduction

Nitrogen removal during wastewater treatment emits nitrous oxide (N_2O) and nitric oxide (NO) (Colliver & Stephenson 2000; Kampschreur et al. 2009b). N_2O is an important greenhouse gas with a global warming potential of 298-CO_2 equivalents (IPCC 2013), which may contribute significantly to the carbon footprint of wastewater treatment plants (WWTPs) (Daelman et al. 2013b; Rodriguez-Caballero et al. 2015). NO is a very reactive gas that is involved in different physiological reactions in living organisms and becomes toxic at certain concentrations (Blaise et al. 2005). Both N_2O and NO contribute significantly to the ozone layer depletion (Kramlich & Linak 1994; Ravishankara et al. 2009). The importance of studying the emissions of these gases from wastewater treatment lies on their environmental impact and their potential function as useful indicators of process disturbances (Butler et al. 2009). Understanding the responsible mechanisms involved during the production of N_2O and NO, as well as knowing the factors that influence these emissions is essential to minimize their emissions and to reach a better process operation.

There is increasing evidence that the first step of nitrification (the conversion of ammonia to nitrite) conducted by ammonium oxidizing bacteria (AOB), is the major contributor to N_2O and NO emissions in the biologically mediated wastewater treatment processes (Ahn et al. 2011; Rodriguez-Caballero et al. 2013). AOB can produce N_2O and NO via two known pathways: the nitrifier denitrification and the hydroxylamine oxidation pathway. The first one is favoured by limited oxygen conditions (Goreau et al. 1980; Tallec et al. 2006) and the presence of nitrite (Colliver & Stephenson 2000). The second is enhanced at high ammonium oxidation rates (Wunderlin et al. 2012). Moreover, other factors such as pH (Law et al. 2011), transient anoxic-aerobic conditions (Yu et al. 2010), salinity (Zhao et al. 2014) or aeration intensity (Castro-Barros et al. 2015) have been reported to affect N_2O and NO emissions from nitrifying systems.

A lot of studies focused on the emissions of N_2O and NO for high strength ammonium wastewater, typically the reject water from the anaerobic digester

(Kampschreur et al. 2008b; Schneider et al. 2013; Stuven & Bock 2001). The removal of nitrogen from such wastewater via nitrite has been gaining importance over the last years since it allows substantial energy (less aeration) and chemical savings compared to conventional processes over nitrate. However, partial nitrification systems used for the treatment of high strength wastewater have been shown to produce higher N_2O and NO emissions compared to full nitrification reactors (Ahn et al. 2011; Rodriguez-Caballero et al. 2013) probably because of the high accumulation of nitrite in those systems and also the high ammonium conversion rates.

Relatively little research has been devoted to N_2O and NO emissions from systems treating low strength wastewater. Some studies focused on N_2O emissions from nitrification or combined nitrification-denitrification systems, without measuring NO (Rodriguez-Caballero et al. 2014; Tallec et al. 2006). Kampschreur et al. (2008a) is one of the few studies where NO was measured together with N_2O in a nitrifying culture. They found that N_2O and NO emissions are linearly proportional to the nitrite increase. In systems dealing with low-strength wastewater, the accumulation of nitrite may happen depending on the operating conditions and loading rates of the plant at certain moments. Occasional nitrite accumulation was also put forward as the cause for N_2O emissions from a full-scale municipal (low strength) wastewater treatment plant (Daelman et al. 2015). Moreover, the application of autotrophic nitrogen removal via nitrite for low strength ammonium wastewater is gaining interest since this technology can contribute to energy-positive WWTPs (De Clippeleir et al. 2011; Kartal et al. 2010). However, there is a lack of studies assessing the potential N_2O and NO emissions in these systems where nitrite can accumulate. Besides, the effect of nitrite on NO in nitrifying cultures adapted and non-adapted to nitrite is not well understood yet. Thus, further research is needed regarding the N_2O and NO emissions during nitrification of domestic wastewater and the effect of nitrite accumulation on the N_2O and NO emissions in these systems.

The present study explores the effect of nitrite on the N_2O and NO emissions during the full-nitrification of low strength synthetic ammonium wastewater

through dedicated experiments. The effect of nitrite shocks as well as step-wisely increasing nitrite concentrations in the reactor on the emissions of both gases was assessed to gain understanding of the mechanisms producing N_2O and NO and to fully capture the overall emission dynamics of both gases.

3.2 Materials and methods

3.2.1 Reactor operation

A sequencing batch reactor (SBR) of 8 L was inoculated with activated sludge from the municipal WWTP of Girona (Spain) and was operated during 5 months, obtaining a nitrifying mixed bacterial culture. The SBR was operated in 6-h cycles: 240 min of continuous feeding and aeration, settling during 100 min and 20 min for decanting. Four litres of synthetic wastewater were fed in each cycle, resulting in a hydraulic retention time (HRT) of 12 h. The influent ammonium concentration was set low (50 mg NH_4^+-N L^{-1}) and no temperature control was established in the reactor. Dissolved oxygen (DO) was controlled with a programmable logic controller (PLC) between 2.0 and 2.5 mg O_2 L^{-1} by adjusting the amount of air and nitrogen in the added gas stream, of which the total flow was kept constant at 3.5 L min^{-1}. The pH was controlled between 6.7 and 7.0 by adding 1 M $NaHCO_3$.

The composition of the synthetic wastewater used as influent consisted of: 282.1 mg NH_4HCO_3 L^{-1}, 87.8 mg KH_2PO_4 L^{-1}, 110.0 mg K_2HPO_4 L^{-1} and 2 mL L^{-1} of a trace element solution that included (g L^{-1}): 1.25 EDTA, 0.55 $ZnSO_4 \cdot 7H_2O$, 0.40 $CoCl_2 \cdot 6H_2O$, 1.27 $MnCl_2 \cdot 4H_2O$, 0.40 $CuSO_4 \cdot 5H_2O$, 0.05 $Na_2MoO_4 \cdot 2H_2O$, 1.37 $CaCl_2 \cdot 2H_2O$, 1.25 $FeCl_3 \cdot 6H_2O$, 44.4 $MgSO_4 \cdot 7H_2O$.

The batch tests were conducted when the reactor had been operating for 4 months and it showed stable operation, with all the ammonium from the influent converted to nitrate without accumulation of nitrite (detailed cycle analysis and nitrogen balance presented in Appendix A3.1).

3.2.2 Analysis and measurements

Cycle studies were carried out on a weekly basis to assess the reactor performance. Samples were taken along the cycle and immediately filtered

through disposable Millipore filter units (0.22 μm pore size) and then analysed to determine the NH_4^+-N, NO_2^--N and NO_3^--N concentrations by ion chromatography (ICS5000, DIONEX). Mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were determined according to the standard methods (APHA 1998). During the batch tests the averaged MLSS and MLVSS in the reactor were 0.63 ± 0.09 g MLSS L^{-1} and 0.50 ± 0.05 g MLVSS L^{-1}, respectively.

N_2O and NO were monitored online in the gas phase every 5 seconds during the batch tests. N_2O was analysed with the infrared gas analyser Servomex 4900 (Servomex Group Ltd. East Sussex, UK) and NO was measured via the chemiluminescence gas analyser CLD64 (Eco Physics. Dürnten, Switzerland).

3.2.3 Batch tests

All batch tests were conducted in the SBR. After the decanting phase of the cycle previous to the batch test, the feeding pump was disconnected and the desired addition of ammonium and/or nitrite started. The pH and DO were kept at the same levels as normal reactor operation (described in section 3.2.1). After each test, the SBR operation was kept normal at least for 24h before another test was conducted.

a) Intermittent ammonium feeding with nitrite pulse additions

An experiment was designed to assess the effect of nitrite on N_2O-NO emissions in the absence of ammonium oxidation. A pulse of nitrite of 10 mg NO_2^--N L^{-1} (reactor concentration) was added without ammonium feeding. Once nitrite was completely oxidized to nitrate, ammonium feeding was re-established at a rate of 0.833 mg NH_4^+-N min^{-1}. This test was repeated twice.

b) Continuous ammonium feeding with nitrite pulse addition

Four sets of tests were conducted to check the effect of 4 different nitrite concentrations (5, 10, 25 and 50 mg NO_2^--N L^{-1}), added as pulses, on the N_2O-NO emissions. During all the tests, ammonium was fed continuously at a rate of 0.833 mg NH_4^+-N min^{-1} and 3 to 4 pulses of nitrite (to provide the desired concentration of mg NO_2^--N L^{-1} in the reactor, see Table 3. 1) were added.

Table 3. 1 Nitrite pulse and step-wise increase tests performed and the nitrite concentrations assessed.

Nitrite pulse tests			Step-wise nitrite increase tests	
Batch test	Theoretical NO_2^- concentration (mg NO_2^--N L^{-1})	Number of pulses performed	Batch test	Step-wisely increasing nitrite concentration (mg NO_2^--N L^{-1})
Pulse_5	5	4	Step_10[b]	0→10→20→30
Pulse_10	10	3	Step_25	0→25→50→75
Pulse_25	25	3	Step_50[b]	0→50→100→150
Pulse_50[a]	50	3		

[a] The 3 pulses were performed in 2 separated batch tests due to the length of the test
[b] Each batch test was done in duplicates

c) Continuous ammonium feeding with continuous nitrite addition

Three sets of experiments where the nitrite in the reactor was increased in step-wisely mode (0 till 30; 0 till 75; and 0 till 150 mg NO_2^--N L^{-1}) were conducted. Firstly, the nitrite consumption rate was determined from the tests with nitrite pulse addition (section 3.2.3*b*) (see Appendix A3.2). The desired constant nitrite concentration was achieved by adding nitrite to the SBR until the desired concentration and keeping this concentration by adding nitrite continuously to the reactor at the same rate as it was being consumed by the nitrite oxidizing bacterial community. Each concentration of nitrite tested was maintained in the reactor for a period of 30 to 60 min. During all the tests, ammonium was fed continuously at a rate of 0.833 mg NH_4^+-N min^{-1}.

Table 3. 1 summarizes the sets of tests performed with pulse and continuous nitrite additions (described in section 3.2.3*b* and 3.2.3*c*) and the different nitrite concentrations present inside the reactor.

3.2.4 Calculations

The specific N_2O and NO emission rates were calculated as:

$$r_{N_{(2)}O} = \frac{C_{N_{(2)}O} \cdot Q_G}{M_{biomass}} \left(gN\, gMLVSS^{-1}\, h^{-1} \right)$$

$C_{N_{(2)}O}$: N_2O-N or NO-N concentration in the gas phase (g N L^{-1}).

C_{N_2O} (ppmv)·10^{-6}·0.0409 (mol L^{-1} at 25 °C and 1 atm)·28 (g N mol^{-1}).

C_{NO} (ppmv)·10^{-6}·0.0409 (mol L^{-1} at 25 °C and 1 atm)·14 (g N mol^{-1}).

Q_G : incoming gas flow rate into the reactor (L h^{-1}).

$M_{biomass}$: total biomass in the reactor (g MLVSS).

The N_2O-N and NO-N emission factors (*EF*) per ammonium converted during an interval t_n-t_0 (h) with constant ammonium addition were obtained as:

$$EF_{N_2O} = \frac{\sum_{i=1}^{i=n} C_{N_2O}(t) \cdot Q_G \cdot (t_i - t_{i-1})}{C_{NH_4^+}^{t_0} \cdot V_R^{t_0} + C_{NH_4^+}^{inf} \cdot Q_L \cdot (t_n - t_0) - C_{NH_4^+}^{t_n} \cdot V_R^{t_n}} \cdot 100 \ (\%, w/w)$$

The numerator denotes the N_2O or NO emitted in mg N during an interval t_n-t_0 chosen, which calculation was made by adding the emissions measured every 5 seconds with the analyser (concentration measured x gas flow x 5 s). The denominator corresponds to the NH_4^+ consumed during the mentioned interval t_n-t_0 in mg N, and obtained from mass balances (initial NH_4^+ + NH_4^+ that enters during t_n-t_0 - final NH_4^+ in the reactor = NH_4^+ consumed).

$C_{NH_4^+}^{t_0}$, $C_{NH_4^+}^{inf}$, $C_{NH_4^+}^{t_n}$: concentrations of NH_4^+-N at t_0, in the influent and at t_n (mg N L^{-1}).

$V_R^{t_0}$, $V_R^{t_n}$: liquid volume of the reactor at t_0 and t_n (L).

Q_L : incoming liquid flow rate into the reactor (L h^{-1}).

3.3 Results and discussion

3.3.1 N_2O and NO emissions during nitrification of low strength wastewater during normal reactor operation

During normal reactor operation, only ammonium was fed into the reactor with a loading rate of 0.10 g NH_4^+-N L^{-1} d^{-1}. All the incoming ammonium was converted to nitrate without accumulation of ammonium or nitrite. The N_2O and NO emissions during normal reactor operation were 0.16 ± 0.11% of the ammonium converted being emitted as N_2O-N and negligible NO emissions. These results are comparable to the ones found in literature for systems treating low strength ammonium wastewater through nitrification only or combined nitrification-denitrification (Table 3. 2).

Table 3. 2 N_2O/NO emissions at different full-scale and lab-scale systems performing nitrification or nitrification + denitrification of low strength ammonium wastewater. On-line monitoring in all cases.

Reference	Type of process	Influent (mg N L^{-1})	N_2O Emission (%)	NO Emission (%)	Comment
Full-scale					
(Ahn et al. 2010b)[a]	Separate-stage BNR	309 (TKN)	0.01 - 0.03[b]	N.M.	Both aerobic + anoxic tanks
(Ahn et al. 2010b)[a]	Step-feed non-BNR	410 (TKN)	0.37-3.3[b]	N.M.	Aerated tanks
(Foley et al. 2010)	Activated sludge	47-114 (TKN)	0.6-25[c]	N.M.	7 WWTP evaluated
(Rodriguez-Caballero et al. 2014)	Activated sludge	47.9 ± 2.3 (NH_4^+) 61.6 ± 7.8 (TKN)	0.127[e]	N.M.	
(Burgess et al. 2002)	Sequencing batch reactors	38.7 ± 2.1 (NH_4^+) 69.0 ± 4.5 (TN)	6.8[d]	N.M.	
Lab-scale					
(Beaumont et al. 2004)	Continuous nitrifying activated sludge	184-244 (NH_3)	0.08 - 1.17[d]	N.M.	NH_3 shock loads
(Tallec et al. 2006)	Nitrifying activated sludge	30 ± 4 (NH_4^+) 34 ± 0.8 (TKN)	0.10-0.4[c]	N.M.	
(Kampschreur et al. 2008a)	Continuous nitrifying SBR activated sludge	105 (NH_4^+)	2.8[c]	0.03[c]	
This study	Nitrifying system	50 (NH_4^+)	0.16 ± 0.11[c]	Negligible	

BNR: Biological Nitrogen Removal; **TKN**: Total Kjeldahl Nitrogen; **N.M.**: not measured; **TN**: Total Nitrogen

[a] Ahn et al. (2010) evaluated 12 WWTP. This table shows the results for those WWTP with the minimum and maximum N_2O emission found

[b] N_2O/TN removed

[c] N_2O/N removed

[d] N_2O/NH_4^+ loaded

[e] N_2O/TKN removed

The variability of the N_2O emissions is notable, especially for full-scale measurements (N_2O emissions ranging from 0.01 to 25% of N removed, Table 3. 2). Moreover, most of the studies focus on N_2O emissions, while there is a clear lack of data regarding NO emissions for nitrifying systems treating low strength wastewater (Kampschreur et al. (2008a) and our study only).

3.3.2 Effect of nitrite pulses on N_2O and NO production without ammonium feeding

The effect of nitrite pulse addition without ammonium feeding was assessed (Figure 3. 1).

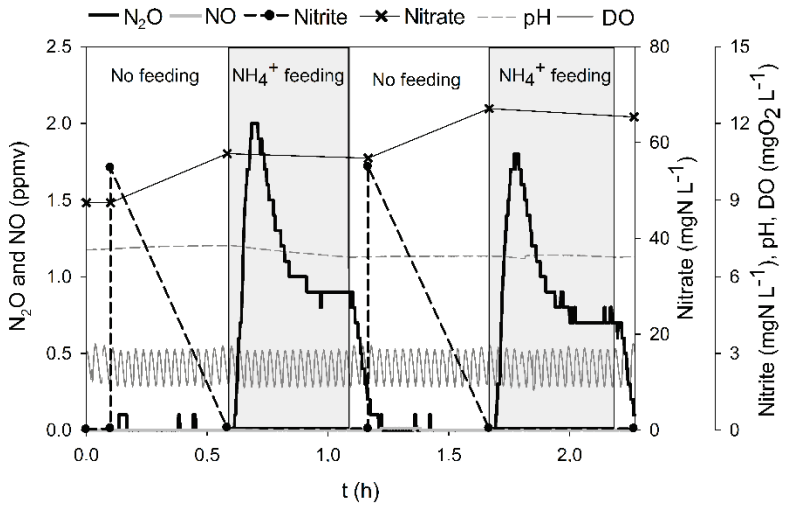

Figure 3. 1 Effect of the nitrite pulse addition without ammonium feeding (white intervals) and effect of the start of the ammonium feeding without addition of nitrite (grey intervals).

When ammonium was not added, nitrite pulses of 10 mg NO_2^--N L^{-1} did not report any emission of N_2O or NO. The small N_2O peaks shown in Figure 3. 1 were not taken into account since these correspond to the lower detection limit of the analyser. Interestingly, the activation of the ammonium feeding in the reactor (grey zones in Figure 3. 1) resulted in an immediate N_2O emission peak (≈ 2 ppmv) even though nitrite had not accumulated. No NO emission was detected in this test. The results demonstrate that the presence of ammonium is required for the production of N_2O.

3.3.3 Effect of nitrite pulses on N_2O and NO production during continuous ammonium feeding

Consecutive pulse additions of nitrite in the tests conducted with continuous ammonium feeding resulted in an increase of N_2O emissions, which decreased to the values before the pulse addition once nitrite was completely oxidized to nitrate (Figure 3. 2). Similar maximum N_2O emission factors with an average of 0.21 ± 0.08% N_2O-N/NH_4^+-N converted were found among the tests conducted with nitrite pulses in the range of 5 to 37 mg NO_2^--N L^{-1}. When 50 mg of NO_2^--N L^{-1} was supplied (Figure 3. 2D), the N_2O emission increased to a mean value of 0.90 ± 0.27% N_2O-N/NH_4^+-N converted.

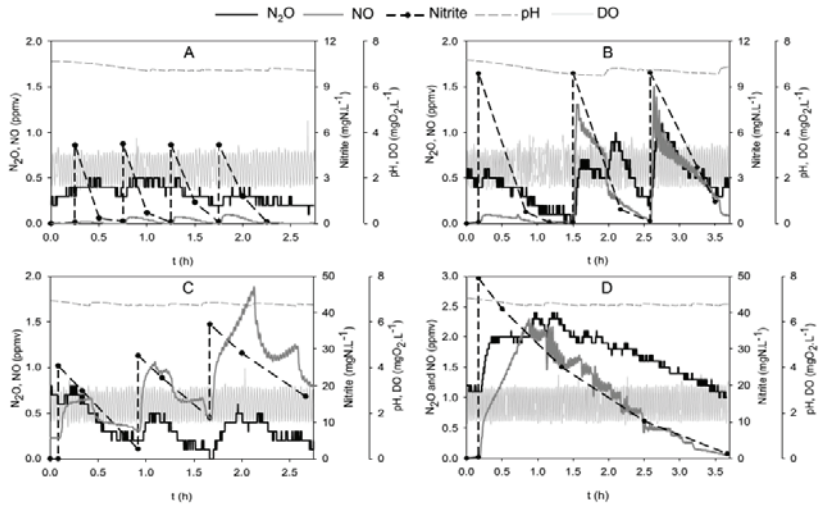

Figure 3. 2 Effect of nitrite pulses on the N_2O and NO production: A) Test performed with pulses of 5 mg NO_2^--N L^{-1}, B) 10 mg NO_2^--N L^{-1}, C) 25 mg NO_2^--N L^{-1} and D) 50 mg NO_2^--N L^{-1}.

NO emissions also increased in response to nitrite pulses (Figure 3. 2). The emission was practically constant for the range of nitrite pulses added (0.11 ± 0.12% NO-N/NH_4^+-N converted). In conclusion, the production of NO needs ammonium (no NO emission detected when supplying only nitrite, white zones in Figure 3. 1), but also nitrite in excess (Figure 3. 2). This clearly discards the N_2O and NO production from heterotrophic denitrification in this reactor, since ammonium is the source for the production of both gases. This

is in accordance with the findings from Kampschreur et al. (2008a), stating that AOB are the main responsible organisms for the production of N_2O and NO in aerobic nitrifying systems treating low strength ammonium wastewater.

Remarkably, the emission of NO was substantially larger during the second nitrite pulse added compared to the emission detected during the first addition (Figure 3. 2A, B and C). This behaviour was observed for NO during all the tests with repeated addition of pulses at the same concentration, but not for N_2O. It seems that short time exposure to nitrite influences the NO emission and indicates that nitrite prompts the mechanism responsible for NO production, which suggests that the nitrifier denitrification pathway significantly contributes to the formation of NO. A possible explanation for this behaviour could be related to the expression of the gene nitrite reductase (*nir*), responsible for the codification of the enzyme NirK, which carries out the reduction of nitrite to NO in AOB. Nitrite has been reported to improve the activity of NirK (Beaumont et al. 2004), which could stimulate the production of NO after the first pulse of nitrite. On the other hand, Kwiatkowski and Shapleigh (1996) reported that the presence of relatively small (non-cytotoxic) NO concentrations result in an enhanced expression of *nir*. Likely in the present study, the supply of nitrite during the first pulse improves the enzymatic activity for the reduction of nitrite to NO, either by the formation of NO or the addition of nitrite itself.

3.3.4 Effect of step-wisely increasing nitrite concentrations on N_2O and NO production

Nitrite was continuously supplied at the same rate as the nitrite consumption rate in the reactor in order to maintain a constant desired concentration (see Appendix A3.3). The nitrite consumption rate remained practically constant (at 35.9 ± 4.9 mg N $(g\ MLVSS)^{-1}\ h^{-1}$, see Appendix A3.3, Figure A3. 3) even with increasing nitrite concentrations, suggesting that the NOB activity was not affected by the increased nitrite concentration and the bacteria were oxidizing nitrite at their maximum rate. This is in accordance to the review of Painter (1970), where much higher nitrite concentration than in the present study

(>1000 mg NO_2^--N L^{-1}) were necessary to partially inhibit the nitrite oxidizers. Step-wisely increasing nitrite concentrations up to 100 mg NO_2^--N L^{-1} resulted in increasing N_2O emissions (Figure 3. 3). For higher nitrite concentrations, the N_2O concentration slightly decreased and remained constant (at around 1 ppmv) (Figure 3. 3C). NO emissions increased with increasing nitrite concentrations in the reactor, surpassing the N_2O emissions for nitrite concentrations around 50 mg NO_2^--N L^{-1} or higher. The NO emission profile differed from the N_2O emission profile in the fact that NO not only increased with increasing nitrite concentration but also with increasing exposure to a given nitrite concentration (Figure 3. 3). Furthermore, the remarkable difference between N_2O and NO emissions at high nitrite concentrations (NO emission up to 8 times higher than N_2O emission, Figure 3. 3C) strengthens the strong effect of nitrite on the NO emissions already observed for the nitrite pulse addition tests (Figure 3. 2).

The sharp build-up of NO in the system with increasing nitrite concentration (Figure 3. 3B and C) suggests that nitrite is the source for the accumulated NO. This abrupt NO formation could be attributed to biological or abiotic formation. In a medium rich in nitrite, NO could be produced chemically from the disproportionation of nitrous acid (HNO_2) (Udert et al. 2005) or from the reaction between nitrite and Fe (II) (Kampschreur et al. 2011). The highest nitrite concentration in the reactor was 150 mg NO_2^--N L^{-1} and the pH was controlled between 6.7 and 7.0. Under these conditions, HNO_2 inhibition of ammonium or nitrite oxidation processes is unlikely (Anthonisen et al. 1976). The iron source added in the medium is Fe (III) in a very low concentration (0.00925 mM). Fe (II) could be produced from the reduction of Fe (III) under anoxic conditions (Weber et al. 2006), such as during the settling period of the SBR cycle. The formation of NO from nitrite and the potential Fe (II) formed would give Fe (III), stopping the reaction when the Fe (II) is depleted (Kampschreur et al. 2011), while the NO formed would act as an intermediate in further conversion to N_2O.

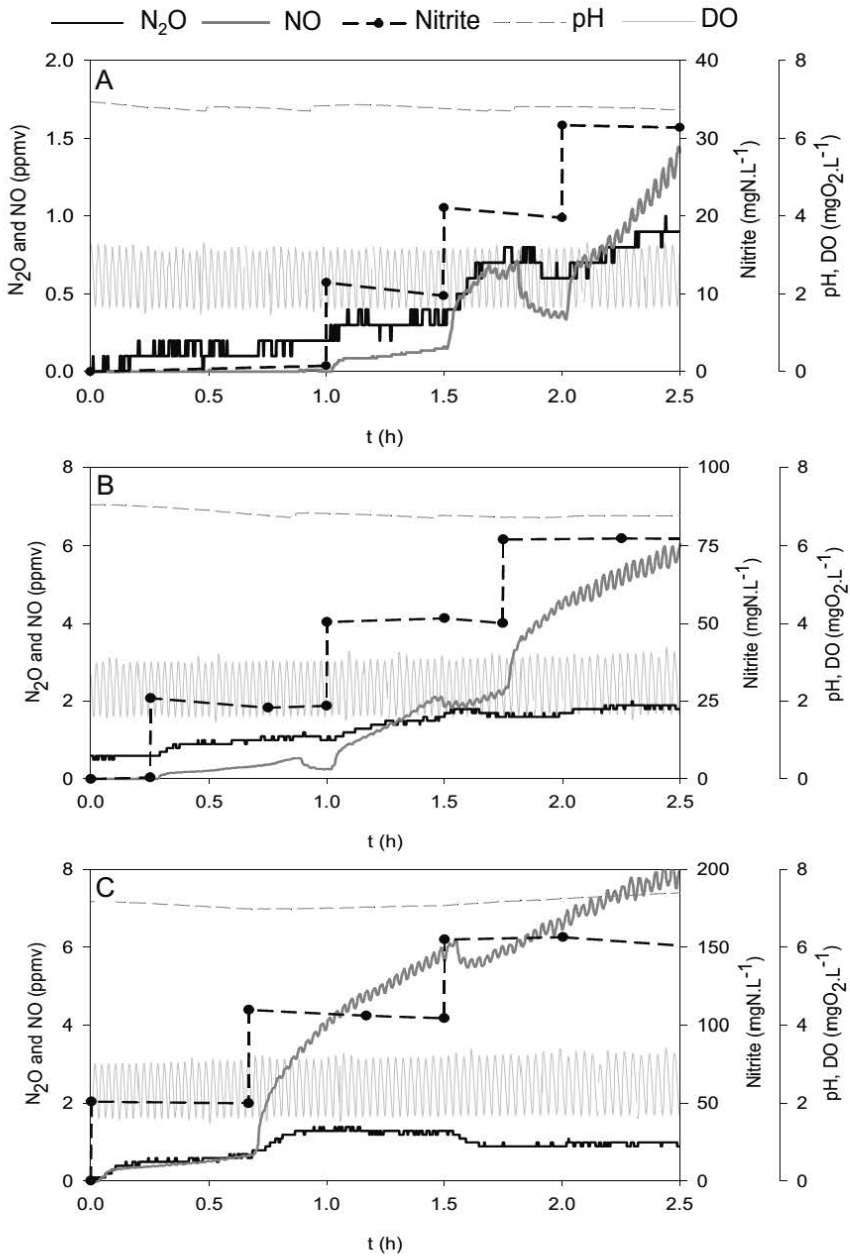

Figure 3. 3 Effect of the stepwise increase of the nitrite concentration on the N_2O and NO emissions: A) test with 10→20→30 mg NO_2^--N L^{-1}; B) 25→50→75 mg NO_2^--N L^{-1}; C) 50→100→150 mg NO_2^--N L^{-1}. Note that the y axis in A is 4 times smaller than in B and C.

However, in the present study the NO and N_2O emissions do not follow the same pattern (NO accumulates continuously and N_2O reaches a stable or even decreasing emission at a given nitrite concentration (Figure 3. 3B and C)). Besides, the formation of NO does not stop for high nitrite concentration in the medium, although the Fe (III) concentration is very low and could only yield a very low Fe (II) concentration (for the experiments of Kampschreur et al. (2011) the Fe (II) concentration is more than 1000 times higher than in the present study), resulting in electron donor limitation for the NO and N_2O formation. For these reasons, chemical NO production is considered irrelevant in the present study. The NO increase with increasing nitrite concentration could be explained from a microbiological point of view. It is known that the responsible enzymes for the reduction of nitrite to NO (nitrite reductase, NirK) and for the reduction of NO to N_2O (nitric oxide reductase, NOR) have independent functionality (Glockner et al. 1993). Thus, the production of NO and N_2O can be assumed as independent, which explains the different patterns observed during the emissions of both gases (Figure 3. 3). Moreover, nitrite and/or NO may act as a substrate inhibitor for NOR at certain concentrations (Cua & Stein 2011; Duarte et al. 2014; Girsch & deVries 1997), while nitrite was observed to improve the levels of NirK (Cua & Stein 2011; Stein & Arp 1998; Yu & Chandran 2010) and nitrite and/or NO to enhance the expression of NirK (Beaumont et al. 2004) (already discussed in section 3.3.3). Therefore, the more nitrite is in the system, the more NO is produced and the formation of N_2O is slowed down, resulting in NO accumulation.

3.3.5 Low vs. high strength wastewater

Increasing nitrite concentration in the SBR resulted in increasing N_2O and NO production rate per ammonium converted (Figure 3. 4). The NO production substantially increased when nitrite concentration was higher than 50 mg NO_2^--N L^{-1} resulting in higher emissions for NO than for N_2O in those tests.

These results differ from the study of Law et al. (2013b) where in an enriched AOB culture treating reject wastewater (1g NH_4^+-N L^{-1}) and always exposed to high levels of nitrite, an increase in nitrite concentrations from 50 to 500

mg NO_2^--N L^{-1} resulted in a gradual decrease in N_2O emissions. Additionally, Pijuan et al. (2014) did not find any effect on the N_2O emissions in a granular sludge airlift reactor of 150 L performing full partial nitrification when exposed to high nitrite concentrations (368-740 mg NO_2^--N L^{-1}). This highlights the capability of AOB to adapt to different environments and reactor conditions (high vs. low nitrite concentrations) responding differently in terms of emissions when being exposed to the same nitrite concentration if the culture was adapted to high or low nitrite concentrations.

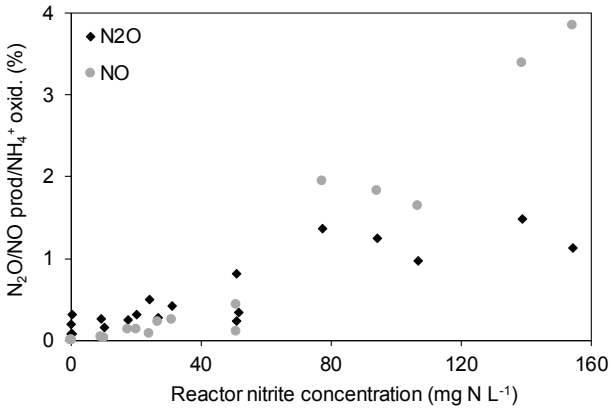

Figure 3. 4 N_2O and NO production per ammonium converted during stepwise increase of nitrite concentration in the reactor.

This adaptation is also shown by the nitrification activity. There is a significant reduction on the nitrification activity in the present study when the bacteria are exposed to concentrations higher than 50 mg NO_2^--N L^{-1} (average of 12.6 ± 1.1 mg NH_4^+-N (g MLVSS)$^{-1}$ h^{-1} consumed when exposed at 0 to 50 mg NO_2^--N L^{-1} and 5.1 ± 0.7 mg NH_4^+-N (g MLVSS)$^{-1}$ h^{-1} when exposed at 150 mg NO_2^--N L^{-1}) (Appendix A3.3, Figure A3. 3), while for those cultures adapted to high nitrite concentrations, the AOB activity was not affected (Law et al. 2013b). Thus, high nitrite concentrations affect the AOB activity when the bacteria are not acclimated to nitrite. It is well known that nitrification is inhibited by HNO_2 (Anthonisen et al. 1976), but minimal formation of HNO_2 is expected in the present study since the pH is controlled between 6.7 and 7.0 (mentioned in section 3.3.4 already). Moreover, Law et al. (2015) showed that the minimal formation of HNO_2 in a lab-scale partial nitrification reactor operated with a

nitrite concentration from 500-1000 mg NO_2^--N $L^{-1}$and controlled pH at 6.2 did not affect the nitrifying activity. Thus, it is hypothesized that nitrite is the source of the ammonium oxidation inhibition in the present study. This could be related to an enzymatic inhibition. Several studies found a reduction of the levels of *amoA* (gene that encodes the oxidation of ammonium to hydroxylamine) in AOB cultures incubated with high nitrite concentrations (Cua & Stein 2011; Stein & Arp 1998; Yu & Chandran 2010). Note that the ammonium oxidation was recovered after the exposition to nitrite. The bacterial activity at the beginning of each test (before the accumulation of nitrite) was very similar and did not decreased (Appendix A3.3, Figure A3. 3). This reversible inhibition in AOB activity has been shown by other authors (Painter 1970). Moreover, it was assumed that the influence at the enzymatic level was also reversible due to the short exposure time to nitrite (maximum of 3 hours), leaving the reactor under normal operation (without nitrite) for at least 24 hours between consecutive tests, and the recovery of the bacterial activity. Long term contact to nitrite may lead to permanent enzymatic changes allowing the adaptation of the bacteria to nitrite, as shown by Law et al. (2013b) and Pijuan et al. (2014).

Although the decrease in the nitrifying activity (Figure A3. 3) occurs when the NO emission is more significant, at nitrite concentrations higher than 50 mg NO_2^--N L^{-1} (Figure 3. 3C and Figure 3. 4), it is not possible to clearly discern if the actual inhibitor is the nitrite or the produced NO. However, since NO accumulation increases with increasing nitrite concentration (Figure 3. 4), NO is the consequence of increasing nitrite and thus, nitrite could be indirectly linked to the nitrifying inhibition in the hypothetical case that the real inhibitor was the NO.

3.3.6 Main mechanism producing N_2O and NO

It is clear that AOB are the organisms producing N_2O and NO from biological processes in the present study. Regarding the main pathway, aerobic conditions promote the hydroxylamine route (Wunderlin et al. 2012), while nitrifier denitrification is stimulated at high nitrite concentrations and although it is enhanced under limited oxygen concentrations (Tallec et al.

2006) it also occurs under aerobic conditions (up to 3.5 mg O_2 L^{-1}, Peng et al. (2015)). Our findings suggest that during nitrite accumulation, nitrifier denitrification is the main pathway for the N_2O and NO production. All tests assessed with nitrite (pulse addition and step-wisely increasing nitrite concentration) showed an improved production of NO, suggesting that the nitrite added is the precursor for the NO and N_2O production, and not hydroxylamine. NO clearly cannot be neglected as intermediate during nitrifier denitrification, in contrast to other studies (Ni et al. 2014), when studying the N_2O and NO emissions during nitrification of low strength ammonium wastewater. In accordance to our results, Daelman et al. (2015), in a N_2O monitoring campaign from a full-scale municipal WWTP, found nitrifier denitrification the dominant pathway for N_2O emission when nitrite accumulates in the system.

Furthermore, the likely enzymatic inhibition caused by accumulation of nitrite during the step-wisely increasing nitrite concentration experiments strengthens the hypothesis of nitrifier denitrification as main mechanism during accumulation of nitrite. The increase of N_2O production is stopped at 100 mg NO_2^--N L^{-1} and then even reduced at 150 mg NO_2^--N L^{-1} (Figure 3. 3C). At these nitrite concentrations it was discussed that the levels of the gene involved in the hydroxylamine pathway (*amoA*) are reduced (mentioned in section 3.3.5), reducing their activity. Thus, the production of N_2O at these high nitrite concentrations could be linked to nitrifier denitrification.

During normal reactor operation (no accumulation of nitrite), the hydroxylamine oxidation pathway could have played an important role in N_2O formation. Peng et al. (2015) showed in a nitrifying reactor treating high strength ammonium wastewater that the contribution of the hydroxylamine oxidation pathway to the N_2O production was only important at very high oxygen concentrations (3.5 mg O_2 L^{-1}) and very low levels of nitrite (< 10 mg NO_2^--N L^{-1}). In the present study, nitrite did not accumulate during normal reactor operation and the oxygen concentration was not limited (2.0-2.5 mg O_2 L^{-1}), which suggests that the role of hydroxylamine oxidation pathway may have been important during normal reactor operation; note however that N_2O

emissions were very small in this case (0.16 ± 0.11% of the ammonium converted being emitted as N_2O-N) and no emission of NO was detected.

3.4 Conclusions

- Nitrite pulses in a nitrification reactor resulted in an increase in N_2O and NO emissions. These emissions decreased to original levels when nitrite was completely oxidized to nitrate.
- Step-wise addition of nitrite (reactor concentrations from 0 to 150 mg NO_2^--N L^{-1}) during ammonium oxidation caused an increase in the N_2O (from 0.16% to 1.5%) and NO (from negligible values to 3.8%) emission factors (N_2O or NO emitted per NH_4^+-N converted).
- NO emissions were higher than those of N_2O when biomass was exposed to high nitrite concentrations (> 50 mg NO_2^--N L^{-1}).
- The release of the intermediate NO in the nitrifier denitrification N_2O production pathway cannot be neglected.
- High nitrite concentrations showed an inhibitory effect on the nitrifying activity of non-adapted bacterial groups to nitrite, likely due to NO accumulation.

Acknowledgements

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Appendix

A3.1 Stable operation of the reactor

The normal or stable operation of the reactor was reached when all ammonium fed into the reactor was converted to nitrate without the accumulation of nitrite or ammonium.

The results for a typical cycle analysis performed before conducting the tests are presented in this section. The settling and decanting intervals (2 hours) were not considered, but the feeding period (4 hours), where the N_2O and NO batch tests were performed. Figure A3. 1 shows the amount of ammonium, nitrite and nitrate in the reactor at different sampling points along the cycle.

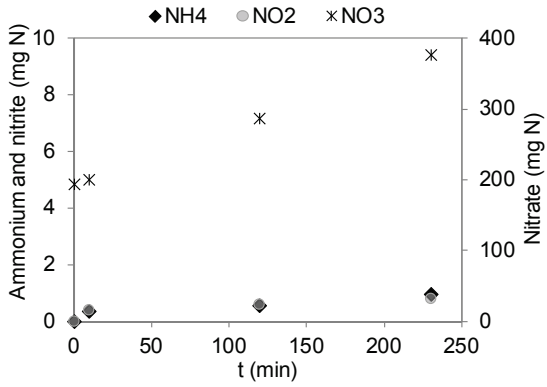

Figure A3. 1. Results from a typical cycle study performed in the SBR before starting the tests.

The nitrogen mass balance of the cycle is presented in Table A3. 1, where all values for the calculation are given. The mass balance was made between time t_1 (start of the cycle) and t_2 (min 230, close to the end of the feeding period of the cycle). The results show that the amount of ammonium fed into the reactor is the same as the nitrate formed (difference of 2% (186-183)/183·100).

Table A3. 1 Nitrogen mass balance for the cycle presented in Figure A3. 1.

Q (L min^{-1})	0.017
t_2-t_1 (min)	230
C_{NH4_inf} (mg N L^{-1})	48.9
M_{NH4_added} (mg N)	187
$M_{NH4}(t_1)$(mg N)	0.00
$M_{NH4}(t_2)$(mg N)	0.95
NH_4^+ consumed (mg N)	186
$M_{NO3}(t_1)$(mg N)	193
$M_{NO3}(t_2)$(mg N)	376
NO_3^- formed (mg N)	183

Where:

Q: liquid flow rate of the influent pump.

C_{NH4_inf}: concentration of ammonium in the influent.

M_{NH4_added}: mass of ammonium added during the interval t_2-t_1.

$M_{NH4}(t_i)$: mass of ammonium in the reactor at time t_i.

$M_{NO3}(t_i)$: mass of nitrate in the reactor at time t_i.

A3.2 Ammonium and nitrite consumption rates during the nitrite pulse tests

The ammonium and nitrite consumption rates were calculated from mass balances between sampling intervals at time t_1 and t_2.

For the ammonium consumption rate (NH_4OR), the expression A3.1 was used:

$$NH_4OR = \frac{M_{NH4}(t_1) + C_{NH4\_inf} \cdot Q \cdot (t_2 - t_1) - M_{NH4}(t_2)}{(t_2 - t_1) \cdot MLVSS} \quad [A3.1]$$

Where:

$M_{NH4}(t_i)$: mass of ammonium in the reactor at time t_i (mg N).

C_{NH4_inf}: concentration of ammonium in the influent (mg N L^{-1}).

Q: liquid flow rate of the influent pump (L d^{-1}).

MLVSS: mixed liquor volatile suspended solids in the reactor (g MLVSS).

The nitrite consumption rate (NO_2OR) was calculated from equation A3.2 for an interval of time t_2-t_1.

$$NO_2OR = \frac{M_{NO2}(t_1) + M_{NH4\_consumed} - M_{NO2}(t_2)}{(t_2 - t_1) \cdot MLVSS} \quad [A3.2]$$

Where:

$M_{NO2}(t_i)$: mass of nitrite in the reactor at time t_i (mg N). This term includes the pulse of nitrite when this is performed.

$M_{NH4\_consumed}$: mass of ammonium consumed (mg N). The ammonium consumed is converted to nitrite and it is included in the mass balance as a nitrite input.

The ammonium and nitrite consumption rates showed in Figure A3. 2 are the averaged values obtained for the calculations among samples taken at different times for each nitrite pulse test following equations A3.1 and A3.2.

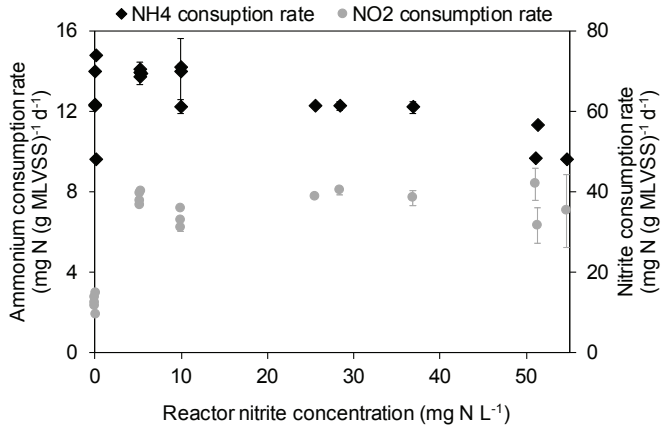

Figure A3. 2 Ammonium and nitrite consumption rates during the nitrite pulse tests. Nitrite concentration refers to the instantaneous nitrite concentration in the reactor after the pulse addition.

A3.3 Ammonium and nitrite consumption rates during step-wisely increasing nitrite concentration tests

The ammonium consumption rate was calculated for the step-wisely increasing nitrite tests with expression A3.1.

For the nitrite consumption rate calculation an additional term was added to the mass balance compared to nitrite pulse addition tests, resulting in

equation A3.3. Nitrite was added in continuous with an additional pump during these tests in order to keep a constant nitrite concentration inside of the reactor. The nitrite flow rate added in continuous was determined from the nitrite consumption rates obtained during the tests with nitrite pulse addition (Figure A3. 2) and the desired nitrite concentration in the reactor.

$$NO_2OR = \frac{M_{NO2}(t_1) + M_{NH4\_consumed} + C_{NO2\_inf} \cdot Q_{NO2\_inf} \cdot (t_2 - t_1) - M_{NO2}(t_2)}{(t_2 - t_1) \cdot MLVSS} \quad [A3.3]$$

Where:

C_{NO2_inf}: nitrite concentration of the nitrite solution added in continuous.

Q_{NO2_inf}: flow rate of the nitrite solution added during time t_2 and t_1.

The results for the ammonium and nitrite consumption rates for the tests with step-wisely nitrite increasing concentration are presented in Figure A3.3.

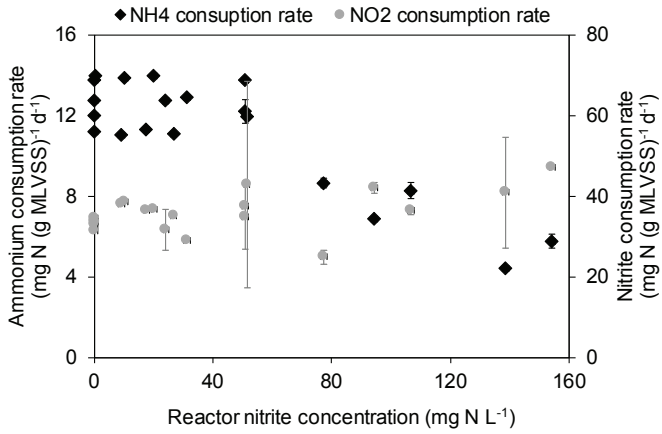

Figure A3. 3 Ammonium and nitrite consumption rates during the stepwise nitrite concentration tests.

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Effect of aeration regime on N_2O emission from partial nitritation-anammox in a full-scale granular sludge reactor

Abstract

N_2O emission from wastewater treatment plants is high of concern due to the strong environmental impact of this greenhouse gas. Good understanding of the factors affecting the emission and formation of this gas is crucial to minimize its impact. This study addressed the investigation of the N_2O emission dynamics in a full-scale one-stage granular sludge reactor performing partial nitritation-anammox (PNA) operated at a N-loading of 1.75 kg NH_4^+-N m^{-3} d^{-1}. A monitoring campaign was conducted, gathering on-line data of the N_2O concentration in the off-gas of the reactor as well as of the ammonium and nitrite concentrations in the liquid phase. The N_2O formation rate and the liquid N_2O concentration profile were calculated from the gas phase measurements. The mean (gaseous) N_2O-N emission obtained was 2.0% of the total incoming nitrogen during normal reactor operation. During normal operation of the reactor under variable aeration rate, intense aeration resulted in higher N_2O emission and formation than during low aeration periods (mean N_2O formation rate of 0.050 kg N m^{-3} d^{-1} for high aeration and 0.029 kg N m^{-3} d^{-1} for low aeration). Accumulation of N_2O in the liquid phase was detected during low aeration periods and was accompanied by a relatively lower ammonium conversion rate, while N_2O stripping was observed once the aeration was increased. During a dedicated experiment, gas recirculation without fresh air addition into the reactor led to the consumption of N_2O, while accumulation of N_2O was not detected. The transition from a prolonged period without fresh air addition and with little recirculation to enhanced aeration with fresh air addition resulted in the highest N_2O formation (0.064 kg N m^{-3} d^{-1}). The results indicate that adequate aeration control may be used to minimize N_2O emissions from PNA reactors.

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4.1 Introduction

The partial nitritation-anammox (PNA) process constitutes a more sustainable alternative for nitrogen removal than conventional nitrification-denitrification over nitrate, since it requires up to 63% less aeration energy, does not require the addition of an external carbon source and the production of sludge and CO_2 emissions are minimal. During partial nitritation, half of the ammonium in the wastewater is oxidized to nitrite by ammonium oxidizing bacteria (AOB) under aerobic conditions and subsequently, anaerobic ammonium oxidizing (anammox) bacteria convert the remaining ammonium and the produced nitrite directly to nitrogen gas. The PNA conversions can be carried out in two separated units as in the SHARON-anammox configuration (van Dongen et al. 2001) or in one reactor unit (Li et al. 2011; Sliekers et al. 2002; Vlaeminck et al. 2009; Wett 2007). The implementation in one reactor is established by controlling the aeration such that AOB and anammox bacteria can coexist. Biofilm reactors are very suitable systems for this purpose, since biofilms have aerobic outer zones, where AOB can grow and consume oxygen; and anoxic inner zones, where anammox bacteria can develop. During the performance of PNA in one reactor unit configuration for granular sludge reactors (Vlaeminck et al. 2008), the bacteria grow in the form of dense, fast-settling granules without carrier material.

Nowadays, general interest does not only focus on effluent quality from wastewater treatment plants (WWTP), but also on the energy consumption and greenhouse gas emissions, aiming at future energy self-sufficient WWTPs with zero carbon footprint (GWRC 2008). Biological processes for nitrogen removal from wastewater produce N_2O, a powerful and stable greenhouse gas with a global warming effect which is 298 times larger than CO_2 over a 100 year time horizon (IPCC 2013). Good knowledge of the pathways and influencing factors for the formation and emission of N_2O is crucial to propose effective strategies that contribute to the minimization of its emissions. N_2O can be formed during nitrification, denitrification and by chemical reactions in WWTPs (Desloover et al. 2012; Kampschreur et al. 2009b). AOB are one of the main responsible groups for N_2O formation during nitrification

(Beaumont et al. 2002; Jiang & Bakken 1999; Lipschultz et al. 1981; Shaw et al. 2006). Two main pathways are known to produce N_2O by AOB: the reduction of nitrite to N_2O (nitrifier denitrification), which is benefited by nitrite accumulation (Colliver & Stephenson 2000) in the system and by limited oxygen concentrations (Tallec et al. 2006); and the oxidation of hydroxylamine (NH_2OH) during ammonia oxidation, which is favoured at high ammonium and low nitrite concentrations and at high nitrogen (ammonium) oxidation rate (Wunderlin et al. 2012). Heterotrophic denitrifiers are also able to produce N_2O since this compound is an obligate intermediate during denitrification. The presence of oxygen (Lu & Chandran 2010) or the nitrite accumulation (von Schulthess et al. 1994) are related to N_2O emissions by heterotrophic bacteria during denitrification. Given that N_2O is not involved in the anammox metabolism (Kartal et al. 2011), it is generally accepted that anammox bacteria do not contribute to N_2O emissions. Very low emissions of N_2O (0.056 ± 0.02% of the total nitrogen load) were observed by Lotti et al. (2014c) in an almost pure anammox culture (98 ± 1%). These N_2O emissions were attributed to a possible side reaction, such as the reduction of NO, an intermediate in the anammox pathway (Kartal et al. 2011) by chemical reactions and/or by other bacteria with the gene to perform such reaction (e. g. AOB or heterotrophic denitrifiers). However, although anammox bacteria could indirectly contribute to N_2O formation in this way, this contribution is expected to be negligible in most practical cases.

The range of N_2O emissions from full-scale WWTP reported in literature is large (0 to 14.6% by Kampschreur et al. (2009b) and 0 to 25% by Law et al. (2012b)). While it is clear that PNA systems result in considerably less energy use related CO_2 emissions than those based on nitrification-dentrification over nitrate, the question remains what is their impact in terms of N_2O emissions and how these emissions are influenced by process design and operation. The aim of this study was to quantify N_2O emissions from a full-scale partial nitritation-anammox reactor, given that little information has been gained on these type of systems up till now. The dynamic reactor behaviour is assessed in detail, while particular attention is paid to the effect

of the aeration pattern on the emission and formation of N_2O, in order to identify possible mitigation measures.

4.2 Materials and methods

4.2.1 Plant and reactor operation

The Olburgen treatment plant (the Netherlands) treats the wastewater from a local potato processing plant and the sludge digestion reject water originating from the municipal WWTP. The industrial wastewater is treated anaerobically in upflow anaerobic sludge blanket (UASB) reactors obtaining biogas for energy production. The sludge from the municipal wastewater treatment is digested and dewatered. The effluents from the UASB reactors and from the dewatering system are combined and treated for nutrient removal. First, phosphorous is eliminated in a PHOSPAQ unit through struvite precipitation and subsequently the nitrogen removal takes place in a one-stage PNA granular sludge reactor (see Appendix, Figure A4. 1). The effluent of the PNA reactor is returned to the municipal WWTP. More details on this plant are given by Abma et al. (2010).

The PNA granular sludge reactor has a liquid volume of 600 m^3 and a headspace volume (gas phase) of 100 m^3. It is operated in continuous mode at a temperature of 37 °C. The reactor is aerated continuously through 64 risers at the bottom of the reactor, which provide mixing. The composition of the aeration gas varies in function of the fraction of fresh ambient air supplied and the fraction of recirculated gas, with lower oxygen content. Figure 4. 1 shows the configuration of the aeration system in the reactor. The amount of fresh air (A) is adjusted to control the effluent quality. When the ammonium concentration increases and the nitrite concentration drops, more fresh air is supplied, thus increasing the dissolved oxygen concentration and enhancing the nitrification activity; when the ammonium concentration becomes lower than its set-point and the nitrite concentration increases, more recirculated gas (D) is provided to the reactor, such that the dissolved oxygen concentration decreases. The total gas flow rate going in and out of the system (A = B in Figure 4. 1) depends on the working capacity of the two compressors

and on the fraction of fresh air added (position of valve A711 in Figure 4. 1). During normal reactor operation, the switch between more and less added fresh air results in intermittent cycles of higher and lower fresh air supply in the system (higher or lower gas flow rate in A and B, aeration). Note that when the speed of the compressors is kept constant and only the valves of fresh air and recirculated gas are changed, the total gas flow rate entering into the reactor (C in Figure 4. 1) is constant, which was the case during the monitoring campaign studied in this chapter.

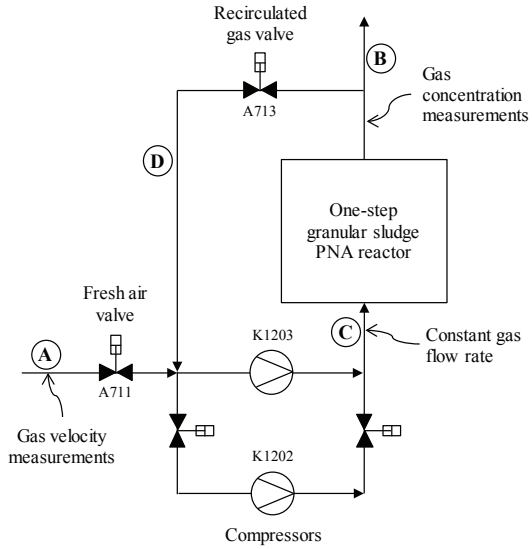

Figure 4. 1 Configuration of the aeration system in the PNA reactor under study in Olburgen.

4.2.2 Nitrogen, phosphorus and COD mass balances over long-term period

The plant performance was monitored over a long-term period (21 months) by setting up nitrogen, phosphorus and COD balances over the PHOSPAQ and PNA units. For this purpose, daily analyses were performed for total Kjeldahl nitrogen (TKN), total phosphorus (TP) and COD in the inlet streams to the UASB reactors as well as the outlet stream from the dewatering unit (streams 1 and 3 in Figure 4. 2, respectively). At the outlet of the PNA reactor (stream 7), weekly analysis of TKN, TP, nitrate and nitrite were conducted, as well as

daily COD analysis. These concentrations and the known online liquid flow rate of streams 1, 3, 4 (the inlet to the PHOSPAQ reactor) and 7 were used to calculate the nitrogen, phosphorus and COD mass balances.

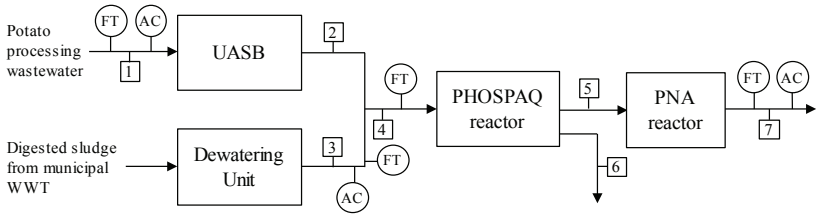

Figure 4. 2 Liquid process streams used for the long-term nitrogen and phosphorus balances for the period January 2010 to September 2011: known weekly concentrations: streams 1, 3 and 7; calculated mass flows: streams 2, 4, 5 and 6. FT (flow transmitter), AC (analysis control).

It was reasonably assumed that no ammonium neither phosphorus removal took place in the UASB reactors and no phosphorus removal occurred in the PNA reactor. It was also supposed that no oxidation of ammonium took place before the PNA unit. The nitrogen removal in the PHOSPAQ unit was calculated from the reaction stoichiometry for struvite precipitation (Eq. 4.1).

$$Mg^{2+} + NH_4^+ + PO_4^{3-} \leftrightarrow MgNH_4PO_4 \cdot 6H_2O \qquad [4.1]$$

The COD removal efficiency in the UASB units was considered to be 90% (Abma et al. 2010).

4.2.3 N_2O emission monitoring campaign

A monitoring campaign was conducted at the PNA granular sludge reactor of the Olburgen sewage treatment plant during one week in summer (22-29 August 2011). The N_2O concentration in the headspace of the reactor was measured with an Emerson MLT4 Rosemount infrared analyser, obtaining on-line data every minute. The ammonium and nitrite concentrations in the effluent of the reactor were measured on-line using an ADI 2019 Process Colorimeters and recorded in the SCADA system every 3-4 minutes. The incoming ammonium concentration was determined off-line, from a grab sample, at 340 g N m^{-3}. The liquid flow rate into the reactor was also recorded continuously in the SCADA system. The outgoing gas flow rate was assumed the same as the incoming gas flow rate, neglecting the consumption and

production of gasses in the reactor. The incoming gas flow was measured at the entrance of the reactor (point A in Figure 4. 1) with a Testo 435-1 hot wire anemometer at different aeration settings. The gas flow rate was obtained from the gas flow velocity and the inner diameter of the pipe (28 cm).

4.2.4 Calculation of N_2O formation rate and dissolved N_2O concentration during normal operation

The emitted N_2O is the result of N_2O formation and subsequent N_2O transfer from the liquid phase to the gas phase (stripping). The N_2O formation rate and the dissolved N_2O concentration during the periods of high aeration were calculated from the measured N_2O gas phase concentration profiles based on the findings of Mampaey et al. (2015).

The gaseous N_2O concentration profile is described by a double exponential profile (Eq. 4.2), which is valid for a N_2O formation rate which is either constant or is approximated by a linear profile in time (see Appendix, section A4.2, Eqs. A4.8 and A4.17, respectively).

$$C_{G,N2O}(t) = a_1(t) + a_2 \cdot exp\left(-\left(a_3 + D_L\right)\cdot t\right) - a_4 \cdot exp\left(-a_5 \cdot t\right) \qquad [4.2]$$

The values of the coefficients a_i are identified by fitting Eq. 4.2 to the measured gaseous N_2O concentration profile. The first term on the right-hand side of Eq. 4.2 (a_1) corresponds to steady state and is related to the N_2O formation rate (Appendix, Eqs. A4.10 and A4.18); a_1 is constant if the N_2O formation rate is so. The remaining coefficients a_i are constant. The second term is related to stripping of accumulated N_2O and the third one to the headspace dilution.

The periods of high aeration were split up into a 'transient part' (HighT) and a 'steady state' part (HighSS). The 'transient part' of the high aeration period corresponds to a switch in the aeration conditions from low to high aeration. For this transient part, the measured gaseous N_2O concentration profile was fitted to Eq. 4.2 assuming a constant coefficient a_1 (Appendix, Eq. A4.8). This is equivalent to assuming a constant (mean) N_2O formation rate, , which is obtained (Eq. A4.13) as:

$$R_{V,N2O}^{HighT} = \frac{a_3 + D_L}{a_3} \cdot \frac{Q_G}{V_L} \cdot a_1 \quad [4.3]$$

From the known gas flow rate Q_G, the liquid flow rate Q_L, the reactor liquid volume V_L and the constants a_i, The corresponding dissolved N_2O concentration profile during the transient part of the high aeration phase, $C_{L,N2O}^{HighT}(t)$, was calculated (Eq. A4.9) as:

$$C_{L,N2O}^{HighT}(t) = \frac{a_1 \cdot Q_G}{a_3 \cdot V_L} + a_2 \cdot \frac{a_5 - (a_3 + D_L)}{a_5} \cdot \frac{Q_G}{a_3 \cdot V_L} exp\left(-(a_3 + D_L) \cdot t\right) \quad [4.4]$$

Under steady state conditions, the gaseous N_2O concentration profile (Eq. 4.2) is simplified to

$$C_{G,N2O}^{HighSS}(t) = a_1(t) \quad [4.5]$$

For the 'steady state' part of the high aeration period, it was found that a_1 was not constant (see further and Appendix, section A4.2). In this sense, the steady state part should rather be seen as a *pseudo* steady state. The gaseous N_2O concentration profile could be fitted to Eq. 4.2 assuming a linear profile of the N_2O formation rate in time, $R_{V,N2O}^{HighSS}(t)$. The latter was then obtained from the measured gas phase concentration $C_{G,N2O}^{HS}(t)$ profile during the steady state part (Eq. A4.24) as:

$$R_{V,N2O}^{HighSS}(t) = \frac{a_3 + D_L}{a_3} \cdot \frac{Q_G}{V_L} \cdot C_{G,N2O}^{HighSS}(t) \quad [4.6]$$

The steady state dissolved N_2O concentration $C_{L,N2O}^{HighSS}(t)$ was then obtained (Eq. A4.27) from:

$$C_{L,N2O}^{HighSS}(t) = \frac{Q_G}{a_3 \cdot V_L} \cdot C_{G,N2O}^{HighSS}(t) \quad [4.7]$$

For the periods of low aeration, it was not possible to obtain an accurate fit for the gaseous N_2O profile to Eq. 4.2. However, the liquid N_2O concentrations at the start (t_{L0}) and the end (t_{Lf}) of the low aeration periods are known, as they are equal to the ones at the end of the preceding and the start of the subsequent high aeration period, which were calculated through Eq. 4.7. As a result, also the N_2O accumulated in the liquid phase during low aeration is known. The mean N_2O formation rate during low aeration ($R_{V,N2O}^{Low}$) could

then be calculated from a N_2O mass balance over the low aeration phase, considering N_2O accumulation in the gas phase and in the liquid phase, as well as gaseous and liquid N_2O emissions:

$$R_{V,N2O}^{Low} = \frac{C_{L,N2O}^{Low}(t_{Lf}) - C_{L,N2O}^{Low}(t_{L0})}{t_{Lf} - t_{L0}} + \frac{C_{G,N2O}^{Low}(t_{Lf}) - C_{G,N2O}^{Low}(t_{L0})}{t_{Lf} - t_{L0}} \cdot \frac{V_G}{V_L} + \frac{1}{V_L} \cdot \frac{1}{t_{Lf} - t_{L0}} \cdot \sum_{i=t_{L0}}^{t_{Lf}} C_{G,N2O}^{Low}(i) \cdot Q_G(i) + \frac{C_{L,N2O}^{Low}(t_{Lf}) + C_{L,N2O}^{Low}(t_{L0})}{2} \cdot \frac{Q_L}{V_L} \quad [4.8]$$

Note that, in Eq. 4.8, the liquid N_2O emissions were approximated based on the average of the N_2O liquid concentrations at the start and the end of the low aeration period. The gas phase N_2O concentrations $C_{G,N2O}^{Low}(t)$ were measured on-line.

Once the mean N_2O formation rate $R_{V,N2O}^{Low}$ was estimated from Eq. 4.8, the N_2O liquid concentration during the low aeration phase could be approximated from:

$$C_{L,N2O}^{Low}(t) = C_{L,N2O}^{Low}(t - \Delta t) + R_{V,N2O}^{Low} - \frac{Q_G}{V_L} \cdot C_{G,N2O}^{Low}(t) \cdot \Delta t - \frac{V_G}{V_L}\left(C_{G,N2O}^{Low}(t) - C_{G,N2O}^{Low}(t - \Delta t)\right) \quad [4.9]$$

which is based on an instantaneous mass balance, in which the liquid phase outflow of N_2O was neglected. Eq. 4.9 can be applied for subsequent time instants, given that the liquid N_2O concentration at the start of the low aeration period is known, being equal to the liquid N_2O concentration at the end of the preceding high aeration period.

4.2.5 Calculation of N_2O formation rate during prolonged aeration

The N_2O formation rate for a dedicated experiment with prolonged aeration after a period without any addition of fresh air was calculated in a different way than for normal operation of the reactor, since a different behaviour of the N_2O emissions was observed, which could not be fit to Eq. 4.2. In this case, after the transition from non-aeration to aeration, the formation rate was assumed equal to the emission rate.

4.3 Results

4.3.1 Nitrogen, phosphorus and COD removal performance

The performance of the PNA granular sludge reactor in terms of nitrogen removal was stable over the period monitored (Figure 4. 3). The nitrogen removal efficiencies for the single PNA unit were calculated through mass balances, obtaining an average value of 92.4 ± 3.2% for the ammonium removal and 74.4 ± 7.1% for the total nitrogen (TN). Figure 3 shows the nitrogen load values from the influent and effluent of the PNA reactor.

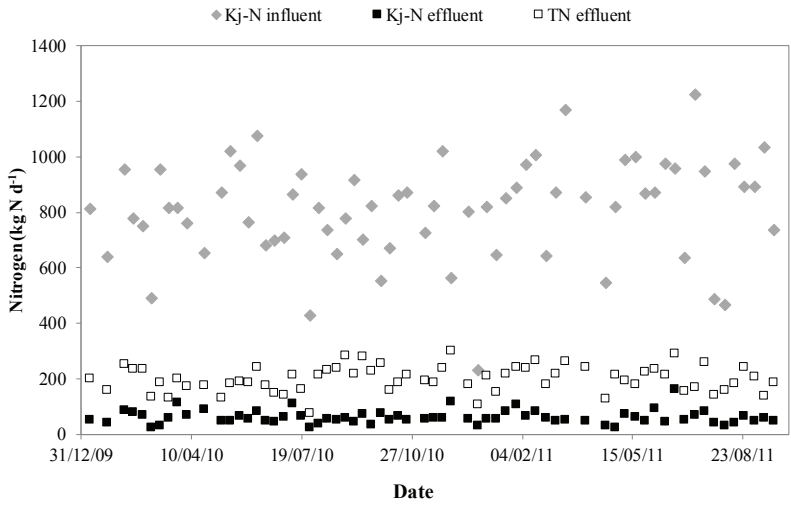

Figure 4. 3 Nitrogen removal in the PNA reactor from January 2010 to September 2011. Kj-N (Kjeldahl nitrogen), TN (total nitrogen).

Including the PHOSPAQ P-recovery unit (as struvite) and PNA together, a mean of 93.0 ± 2.9% of ammonium was removed for the long-term period, and 76.6 ± 6.3% for the TN. These values are slightly higher than the ones reported by Abma et al. (2010) for 2008 (91% and 73% respectively).

From mass balancing, an average COD concentration of 618 ± 98 mg COD L^{-1} over 21-month monitoring period was obtained for the PHOSPAQ influent (stream 4, Figure 4. 2), before nutrient removal. The COD concentration in the PNA effluent (stream 7, Figure 4. 2) was measured, resulting in a mean of 214 ± 83 mg COD L^{-1}, which corresponds to a COD removal efficiency of 65 ± 13%

over the nutrient removal (PHOSPAQ and PNA) units. The COD removal efficiency during the 1-week monitoring campaign amounted to 70 ± 4% on average and is detailed in the Appendix (section A4.3). While it is known that COD oxidation may take place due to the aeration in the PHOSPAQ reactor and by heterotrophic activity in the PNA reactor, the relative importance of these removal mechanisms could not be quantified based on the available measurements.

4.3.2 N_2O emission factor

The mean N_2O-N emission was calculated based on 74 hours of normal operation within the monitored period (22-29 August 2011, Figure 4. 4). The absolute emission for this interval was 61.9 kg N_2O-N and the mean N_2O-N emission factor amounted to 2.0 ± 1.0% of the total incoming nitrogen load of the PNA reactor. A significant difference in the mean emission was found when considering separately the intervals with high and low aeration. For the periods with high aeration (51 hours) 2.5 ± 0.8% of ammonium in the effluent was emitted as N_2O-N, while during the intervals of low aeration (23 hours) the mean emission factor was 1.0 ± 0.3%.

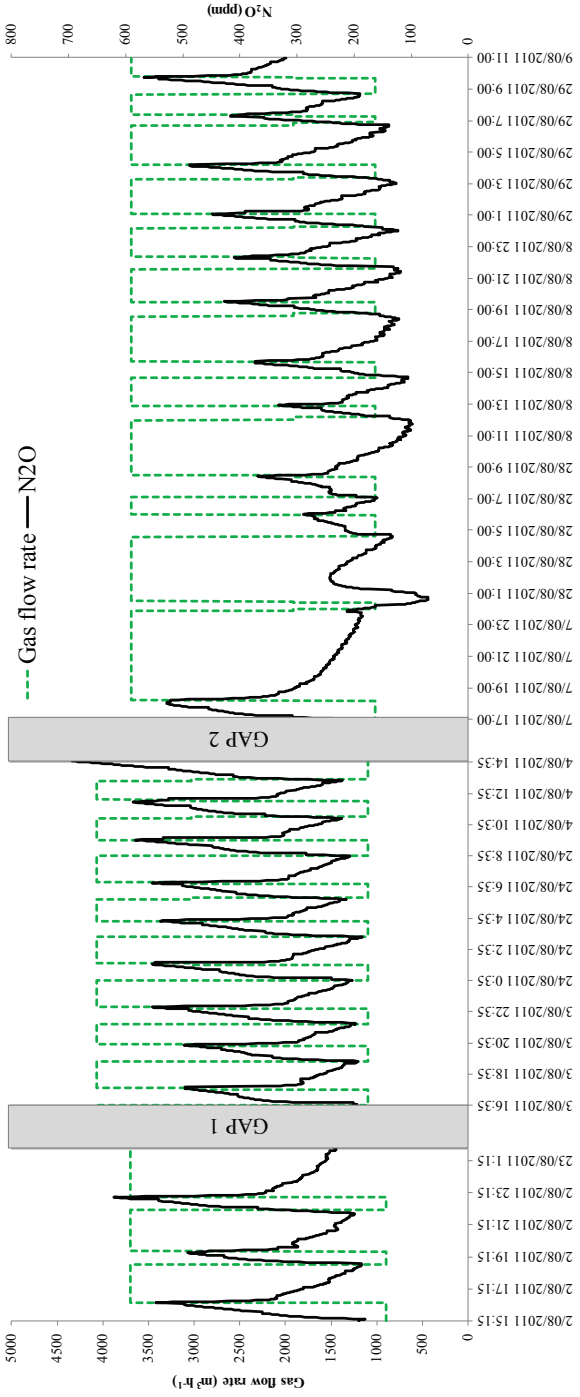

Figure 4. 4 Dynamics of N_2O during normal reactor operation (74 hours in total). GAP 1 (15 h) was dedicated to the calibration of the gas flow rate; in GAP 2 no N_2O data for normal operation were available due to failure of the gas analyzer (44 h), which was followed by a dedicated experiment (30 h, see Figure 4. 7).

4.3.3 Dynamic behaviour during normal operation of the reactor

Focussing on the dynamic reactor behaviour, an interval of 5 hours during normal operation, with two steps of high aeration and two of low aeration was selected (Figure 4. 5). This period was chosen because of its constant liquid flow rate (129 $m^3 h^{-1}$) and because of the intermittent variation of the aeration between the same high and low aeration levels (4041 $m^3 h^{-1}$ and 1088 $m^3 h^{-1}$, respectively) during consecutive cycles.

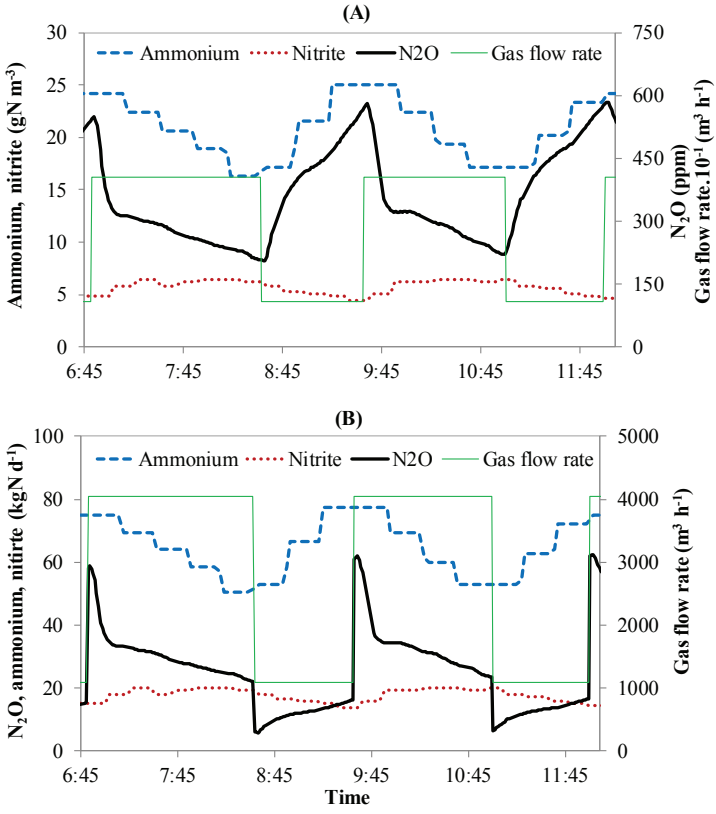

Figure 4. 5 Concentration profiles (A) and mass flow rates (B) of ammonium and nitrite in the liquid effluent and N_2O in the off-gas of the PNA granular sludge reactor, besides the aeration gas flow rate. Data from normal reactor operation on 24 August 2011.

During high (intense) aeration periods, the ammonium concentration decreased from 25 to 17 g N m^{-3}, while the nitrite concentration only slightly increased (4.7– 6.6 g N m^{-3}) (Figure 4. 5A), reflecting a slightly higher increase

in nitrifying activity than in anammox activity. The inverse effect was seen during decreased aeration. As a result, the ammonium removal efficiency in the reactor varied between 93% and 95%.

The emitted N_2O load (Figure 4. 5B) is the result of N_2O formation in the liquid phase and subsequent N_2O transfer from the liquid phase to the gas phase (stripping). The calculated mean N_2O formation rate, as well as the liquid N_2O concentration profile for the four periods of high and low aeration are displayed in Figure 4. 6. The absolute amounts of the N_2O emission and formation and the mean N_2O emission and formation rates are summarized in Table 4. 1. The (time-weighed) mean N_2O formation rate for the two high aeration periods was 0.050 kg N m^{-3} d^{-1} and the mean N_2O formation rate for the two low aeration periods was 0.029 kg N m^{-3} d^{-1}.

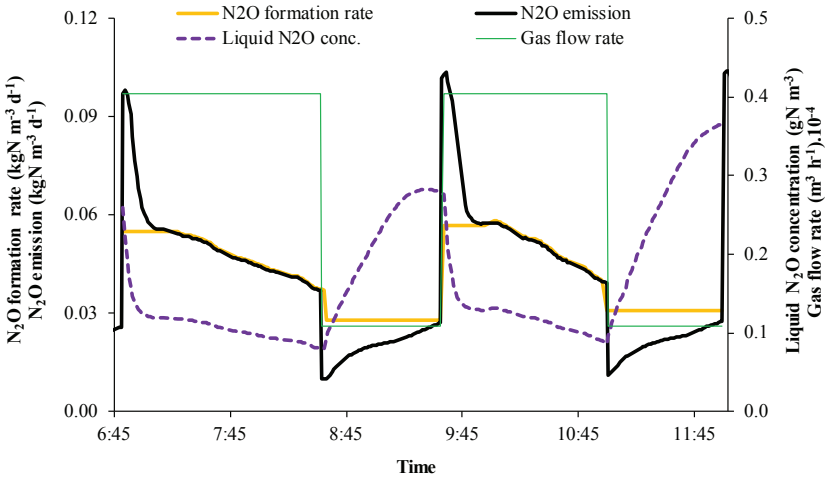

Figure 4. 6 Volumetric N_2O formation rate and liquid N_2O concentration calculated from data from normal reactor operation on 24 August 2011. To allow easy comparison, the N_2O emission rate has been expressed relative to the reactor volume.

Comparing the N_2O formation rate with the N_2O emission rate (Figure 4. 6), it is clear that at the beginning, i.e. during the 'transient part', of the high aeration step the amount of N_2O that was stripped (emission) was about 1.5 times higher than the amount that was formed. During the following 'steady state' part of the high aeration step, the emitted N_2O almost equalled the formed N_2O. Nevertheless, the slight decrease of N_2O in the liquid phase

revealed that the N_2O emission was somewhat higher than the formation during this period. During low aeration periods not all formed N_2O was transferred to the gas phase. This resulted in N_2O accumulation in the liquid phase (Figure 4. 6) during low aeration periods, and an associated increase in the N_2O gas phase concentration (Figure 4. 5A). Note that, overall, for the four intervals considered, the total amount of N_2O emitted equals the amount of N_2O formed (difference of (5.39-5.25)/5.39·100=2.7%, see Table 4. 1), reflecting a closed mass balance.

Table 4. 1 N_2O emission (measured) and formation (calculated) during four selected periods of high and low aeration. Data from normal reactor operation on August 24th 2011 (Figure 4. 5 and Figure 4. 6).

	High aeration (6:50 – 8:30)	**Low aeration** (8:30 – 9:35)	**High aeration** (9:35 – 11:00)	**Low aeration** (11:00 – 12:00)	**Overall** (6:50 – 12:00)
Absolute amount of N_2O emitted (kg N)	2.20	0.514	2.01	0.528	5.25
Mean N_2O emission rate (kg N m^{-3} d^{-1})	0.051	0.020	0.056	0.021	
Absolute amount of N_2O formed (kg N)	2.08	0.719	1.82	0.768	5.39
Mean N_2O formation rate (kg N m^{-3} d^{-1})	0.049	0.028	0.052	0.031	

4.3.4 Dynamic behaviour during prolonged aeration after non aeration

A dedicated experiment was carried out to assess the effect of more severe aeration conditions than during normal reactor operation on the N_2O emission. Therefore, the aeration was changed in 3 senses: duration of the intervals, composition of the gas going into the reactor (more or less fresh air) and sharpness of the transition from low to high oxygen content (anoxic to completely aeration conditions). Firstly, the fresh air supply was stopped during 2 hours (10:30 to 12:30 on August 26th), while a small gas recirculation was kept for biomass suspension and mixing (compressors working at 15% of their capacity). Subsequently, the recirculated gas was stopped and only fresh air was drawn into the reactor during 28 hours (12:30 on August 26th to

16:30 on August 27th) at a relatively constant gas flow rate with a mean value of 4188 $m^3 h^{-1}$ (Figure 4. 7).

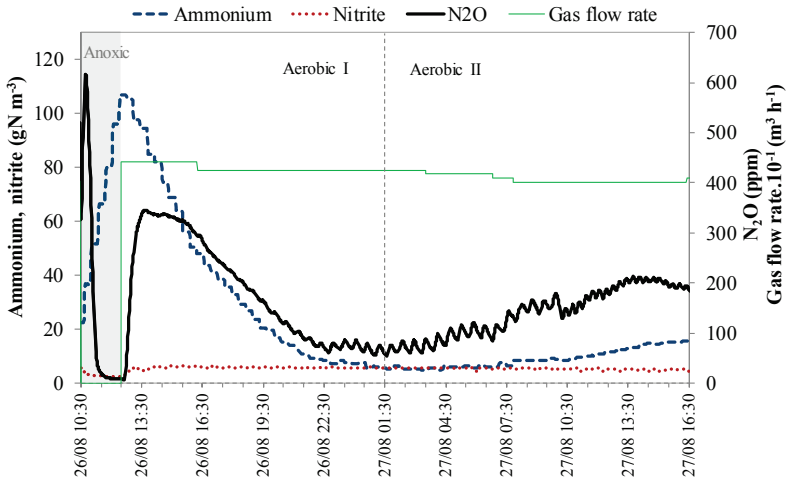

Figure 4. 7 Concentration profiles of N_2O in the off-gas of the reactor, ammonium and nitrite in the liquid effluent and aeration gas flow during a test showing the effect of the switch from no fresh air addition to only fresh air addition into the reactor.

During the period without fresh air addition, ammonium accumulated in the reactor up to 107 g N m^{-3}, which was more than 4 times higher than the maximum ammonium concentration during normal reactor operation, while the nitrite concentration decreased to 2.4 g N m^{-3}. During this period, the ammonium oxidation rate (AOR) decreased due to the low nitrifying and anammox activities from 1.7 kg N m^{-3} d^{-1} during high aeration for normal reactor operation (Appendix, Figure A4. 5) to 0.50 kg N m^{-3} d^{-1} (Figure A4. 6). N_2O accumulated in the beginning of the non-aeration period reaching a peak N_2O gas concentration of 615 ppm, which subsequently dropped to 8 ppm (Figure 4. 7).

The stop of the gas recirculation and the addition of fresh air into the reactor improved the nitrification activity with a high AOR peak of 2.0 kg N m^{-3} d^{-1} in the beginning of this aeration stage (Appendix, Figure A4. 6) and an increase in the nitrite concentration which afterwards remained practically constant during the complete aeration period (5.6 g N m^{-3}) (Figure 4. 7). With the

enhanced aeration, the ammonium concentration decreased to 5.8 g N m^{-3} with a subsequent slight increase at the end of the aeration period (Figure 4. 7) corresponding with a slight decrease in the AOR, from 1.8 kg N m^{-3} d^{-1} to 1.6 kg N m^{-3} d^{-1} (08:00-10:00 on August 27th).

Given that the transient phase during normal reactor operation lasted about 20 minutes (Figure 4. 5) while applying a somewhat lower aeration rate (4014 m^3 h^{-1} during normal operation and 4188 m^3 h^{-1} in this experiment), it was reasonably assumed that, 1 hour after switching on the aeration, steady state was reached during the experiment. The N_2O formation rate in this period was then approximated assuming that it equalled the N_2O emission rate, as it was showed for the steady state part of the high aeration period during normal reactor operation (Figure 4. 6) that these values were very close. As the gas flow rate remains practically constant in the period of high aeration (12:30 on August 26th onwards on Figure 4. 7), the N_2O emission (and formation) rate closely follow the N_2O concentration profile for the mentioned period.

A mean N_2O formation rate of 0.064 kg N m^{-3} d^{-1} was found in the beginning of the aeration interval (13:30-15:30 on August 26th), after which the formation rate was decreasing along prolonged aeration period, to 0.013 kg N m^{-3} d^{-1}. Overall, the mean N_2O formation rate for the whole aeration period was 0.029 kg N m^{-3} d^{-1}, which is very similar to the values calculated for low aeration intervals during normal operation (Table 4. 1). The maximum N_2O concentration reached in the headspace of the reactor after switching on the aeration (337 ppm) was comparable to the concentration after the stripping peak emission during intense aeration intervals during normal reactor operation (318 ppm), this is, the plateau observed just after the rapid decrease in the curve of the N_2O concentration in Figure 4. 5. The emission and formation of N_2O decreased together with the drop in the ammonium concentration. After 12 hours of applying continuous aeration, the N_2O formation increased, accompanied by a slight increase in the ammonium concentration.

4.4 Discussion

4.4.1 N_2O emission from partial nitritation units

Table 4. 2 gives an overview of N_2O-N emission factors obtained from different full-scale partial nitritation configurations. The N_2O emission factor of 2.0 ± 1.0% reached in this study is higher than the one obtained by Kampschreur et al. (2009a) at the same plant in September 2007, being 1.2%. Even though the difference is not statistically significant, the higher influent ammonium load in this study compared to Kampschreur et al. (2009a) (1,053 kg N d^{-1} versus 600 kg N d^{-1}, respectively) could have led to higher emissions. Lotito et al. (2012) reported higher N_2O emissions with higher nitrogen loads. The difference on the dissolved oxygen concentration and/or the aeration scheme and intensity could also explain the difference observed in both studies, but no data are available to make a precise comparison. Furthermore, although the sampling methods were different (grab samples followed by GC analysis for Kampschreur et al. (2009a), online measurements in this study), this was not expected to have a significant effect since the measurement frequency was comparable (every 10 minutes versus every minute, respectively).

Comparing the emission factor from the one-stage partial nitritation granular sludge in this study with the one from two-stage PNA configurations, its value was lower than the 2.3% and 3.7% from the combined SHARON-anammox system (Kampschreur et al. 2008b; Mampaey et al. 2016) and also lower than the emission factor of 5.1-6.6% for the suspended sludge partial nitritation reactor monitored by Desloover et al. (2011).

In general, the emission factor from one-stage PNA processes is expected to be lower than from two-stage PNA processes since the accumulation of nitrite is prevented in the first (low and practically constant for this study, 5-7 mg N L^{-1} during normal reactor operation) and this accumulation can also be correlated to the enhancement of N_2O emissions (Colliver & Stephenson 2000; Kampschreur et al. 2008b; Okabe et al. 2011). However, caution should be taken when interpreting measurement data from literature, since seasonal

variations as well as sampling strategies may influence the N_2O emissions (Daelman et al. 2013a) and should be taken into account when comparing different emission factors.

Table 4. 2 Nitrous oxide emission factor in full-scale partial nitritation units.

Reference	Configuration	Emission factor[a]	Load (kg N d^{-1})	NH_4^+ eff. (g N m^{-3})	NO_2^- eff. (g N m^{-3})	Sampling of N_2O
(Kampschreur et al. 2009a)	One-stage Granular sludge	**1.2**	600[b]	1-2	6-8	grab sample
(Kampschreur et al. 2008b)	Two-stages Suspended sludge partial nitritation + granular sludge anammox	**2.3**	980[c]	78.1-115	4.9-7.2	grab sample
(Desloover et al. 2011)	Two-stages Suspended sludge	**5.1-6.6**	357[b] 489[c]	4.7 ± 5.2	12 ± 6	on-line
(Mampaey et al. 2016)	Two stages (SHARON reactor)	**3.7**	755[b]	-	-	on-line
This study	One-stage Granular sludge	**2.0**	1053[b]	24.5-16.6	4.7-6.6	on-line

[a] N_2O-N % of the total incoming nitrogen load
[b] NH_4^+-N
[c] Kj-N

4.4.2 Effect of anoxic conditions on the dynamics of N_2O

The complete stop in the aeration for a prolonged period (10:30-12:30 in Figure 4. 7) decreased the N_2O concentration inside the reactor and no N_2O emission peak was observed due to stripping of accumulated N_2O once the aeration was put on again (Figure 4. 7). These facts suggest a consumption of N_2O during the period without aeration, for which anoxic conditions can be reasonably assumed, since no fresh air was added, the nitrifying activity was decreased (abrupt accumulation of ammonium, Figure 4. 7) and the anammox activity would produce N_2, helping to avoid the entrance of air from outside. Moreover, the delay observed in the emissions once the aeration starts (12:30 onwards in Figure 4. 7) suggests that the N_2O is formed when the fresh air is added into the reactor, but not during the previous anoxic interval. These results are similar to the ones of Hu et al. (2013), who observed in a lab-scale anoxic/aerobic reactor for nitrogen removal no emission of N_2O during the anoxic phase and to the study of Domingo-Felez et al. (2014), who found

reduction of N_2O during anoxic periods in two lab-scale nitritation-anammox reactors. In those cases, the N_2O emission decrease during anoxic periods was attributed to heterotrophic denitrification. Kartal et al. (2011) showed that N_2O is not an intermediate in the denitrification by anammox bacteria. In this full-scale study, heterotrophic denitrification can be a sink for N_2O, using the available organic matter (see Appendix, Table A4. 1) as an electron donor for the reduction of N_2O to N_2. Note that, such heterotrophic denitrification could be positive from an operational point of view. It was indeed demonstrated by Mozumder et al. (2014) that the maximum nitrogen removal capacity (N_2 production) in granules capable of autotrophic nitrogen removal through partial nitritation-anammox is improved by the presence of relatively small amounts of organic substrate in the influent, because of the incomplete denitrification of nitrate to nitrite, which can be subsequently taken up by anammox bacteria.

4.4.3 Relation between aeration level and N_2O emission and formation

Evidence is available that aerated zones in WWTPs are prone to higher N_2O emissions compared to non-aerated zones or anoxic conditions (Ahn et al. 2010a; Ahn et al. 2010b). This is confirmed by the results from normal reactor operation (Figure 4. 4 and Figure 4. 5) and from the dedicated experiment (Figure 4. 7) obtained in this study, showing higher emissions during periods with higher aeration.

The highest N_2O formation rate was found when recovering from an anoxic period, when a significant accumulation of ammonium (up to 107 g N m^{-3}) took place, to an enhanced aeration interval with only fresh air addition (0.064 kg N m^{-3} d^{-1}, 10:30-12:30 in Figure 4. 7). This coincided with the highest AOR resulting from the high ammonium concentration at the point aeration was resumed (Appendix, Figure A4. 6). The transient from anoxic to aerobic conditions as well as the accumulation or unlimited availability of ammonium was correlated to N_2O formation in other studies (Ahn et al. 2010a; Chandran et al. 2011; Yu et al. 2010). Law et al. (2012a) reported a positive correlation between ammonium oxidation rate and N_2O formation, attributing the N_2O

formation to the hydroxylamine route. Wunderlin et al. (2012) identified high ammonium concentrations and aerobic conditions (high nitrifying activity) as important factors affecting the N_2O formation.

For the normal reactor operation, higher emissions were found during higher aeration periods, which is not only due to enhanced stripping of N_2O from the liquid phase to the gas phase, but also by a higher N_2O formation rate during periods with intense aeration (Figure 4. 6). Low aeration periods resulted in a lower N_2O emission due to its accumulation in the liquid phase and the lower formation rate of N_2O. More specifically, the highest N_2O emission and formation during normal reactor operation were found during the transient from low to high aeration. This study found the highest N_2O formation rate (0.064 kg N m^{-3} d^{-1}) upon the recovery from anoxic conditions (Figure 4. 7). This result, obtained for a mixed culture in a full-scale reactor confirms the findings from Yu et al. (2010), obtained for pure cultures in lab experiments. Moreover, this study demonstrates that higher N_2O formation also results from the transient from low (not anoxic) to high aeration conditions (Figure 4. 5). This indicates that sudden changes from relatively low oxygen content (or anoxic conditions) to relatively high oxygen conditions, favour the formation of N_2O.

The recovery from anoxic and from low aeration periods have the accumulation of ammonium, a higher ammonium oxidation rate, no accumulation of nitrite and transient to a more aeration state (higher oxygen content) in common, which result in higher N_2O formation (0.064 kg N m^{-3} d^{-1} N_2O formation rate upon recovery from anoxic conditions and 0.050 kg N m^{-3} d^{-1} upon transition from low aeration periods during normal reactor operation). These findings suggest that the hydroxylamine pathway is an important contributor in the formation of N_2O during the transient from low aeration (or anoxic) periods to higher aeration intervals. However, heterotrophic denitrification cannot be discarded as an influencing contributor in the formation of N_2O, while the relative importance of both pathways is unknown.

Overall, smother shifts in the aeration pattern are recommended to minimize N_2O emissions, as the transitions during normal reactor operation (less abrupt, Figure 4. 5) reported lower N_2O emissions than the recovery from anoxic conditions (Figure 4. 7).

4.4.4 Influence of prolonged and intense air-fresh addition on N_2O emission and formation

The N_2O emission dynamics were different along the prolonged aeration interval of the dedicated experiment (from 12:30 on August 26th onwards on Figure 4. 7). The prolonged aeration period can be divided in two parts regarding the N_2O emission dynamics: Aerobic I in Figure 4. 7 (discussed before) and Aerobic II in Figure 4. 7. During the second part of the prolonged aeration, the AOR decreased slightly (Appendix, Figure A4. 6) and the ammonium concentration and N_2O emission increased. These facts suggest a decrease in the nitrifying activity, but the underlying reason is not completely clear. Nitrite remains practically constant (Figure 4. 7), which indicates that both AOB and anammox activities are affected to the same extent. Due to the presence of organic matter (Appendix, Table A4. 1) and the higher aeration, heterotrophic activity might have been improved. The increase on the N_2O emissions could possibly be related to this heterotrophic activity, since the high aeration implies higher oxygen level and this was shown to enhance the N_2O production by heterotrophs (Lu & Chandran 2010). The increase on the N_2O emissions could be also an indicator of a bad nitrifying activity, as also pointed out by Butler et al. (2009).

Overall, it seems that N_2O emissions could be minimized by optimizing the aeration regime. The duration of the aeration intervals influences the N_2O emissions. Short aeration periods are recommended to minimize N_2O emissions. This is in accordance with the findings of Domingo-Felez et al. (2014), who found in a lab-scale nitritation-anammox SBR (minute scale in that case and hours in our full-scale study) higher emissions with longer aeration periods and demonstrated that shortening the aeration periods reduces the N_2O emissions. At the same time, as pointed out before, the transition between different aeration regimes should be kept smooth.

4.5 Conclusions

- The overall/average N_2O emission factor of a full-scale one-stage PNA granular sludge reactor was determined at 2.0% of the incoming nitrogen load.
- The N_2O formation rate and the N_2O liquid concentration profiles were calculated from the gas-phase measurements and known operating parameters during the normal operation of the reactor.
- The aeration intensity was an important variable influencing the N_2O emission dynamics: the N_2O emission rate was higher during periods of intense aeration than during low aeration periods, which was not only due to the enhanced N_2O stripping, but also to a larger formation rate.
- Under anoxic conditions, N_2O was consumed in the system.
- Increase in ammonium conversion rates led to increased N_2O formation and emission, therefore, transient aeration patterns from low (or anoxic) to high aeration seem to be negatively affecting the N_2O emission rate and a more continuous aeration might give a lower emission rate.

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Appendix

A4.1 Process layout of the Olburgen sewage treatment plant (Abma et al. 2010)

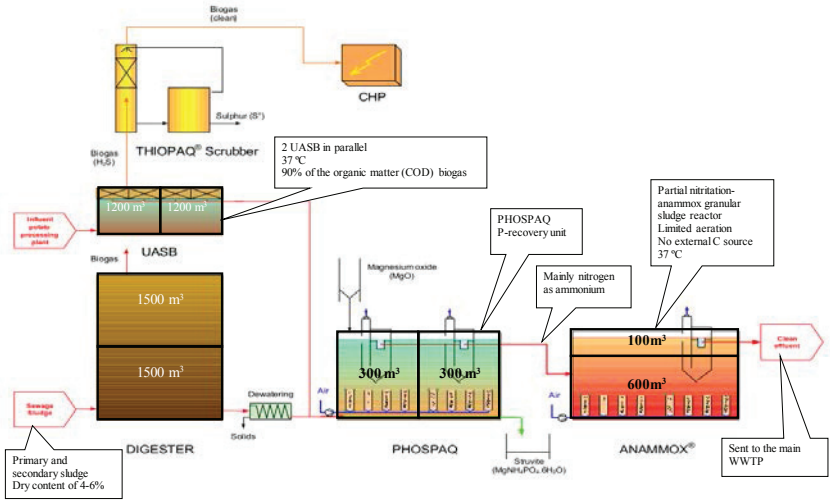

Figure A4. 1 Process layout of the Olburgen sewage treatment plant.

A4.2 Calculation of N_2O formation rate and dissolved N_2O concentration during normal operation

This section contains the theoretical basis for calculating the N_2O formation rate and dissolved N_2O concentration during normal operation. Mass balances are set up over the partial nitritation-anammox reactor, describing its dynamics in terms of the measured gas concentration $C_{G,N2O}(t)$ and the liquid concentration $C_{L,N2O}(t)$, as in Mampaey et al. (2015) (section A4.2.1). These mass balances are solved considering a time varying formation rate $R_{V,N2O}(t)$, a novelty of this study. The findings are subsequently applied to the reactor in this study (section A4.2.2).

A4.2.1 Overall reactor dynamics

The reactor of which the gas phase N_2O concentration is monitored, involves two subsystems. The first subsystem comprises the liquid volume V_L and the

gas bubble volume $V_{G,1}$ of the reactor. The second subsystem concerns the reactor headspace, V_G .

Under the **following assumptions**,

the liquid and gas phase in subsystems 1 and 2 (V_L , $V_{G,1}$, V_G) are **perfectly mixed.**

the **gas phase** (bubbles) in subsystem 1 is in **quasi steady-state,** $\frac{dC_{G,1}(t)\cdot V_{G,1}}{dt}=0$, considering the short gas residence time relative to the time constant for interphase mass transfer and biological conversion rates.

the volumes V_L and V_G are **constant.**

the liquid feed does not contain dissolved N_2O, $C_L^{in}(t)=0$.

the reactor behaviour is described by (Mampaey et al. 2015):

$$\frac{dC_{L,N2O}(t)}{dt}=R_{V,N2O}(t)-(a_3(t)+D_L(t))\cdot C_{L,N2O}(t) \qquad [A4.1]$$

$$\frac{dC_{G,N2O}(t)}{dt}=a_5(t)\cdot\frac{a_3(t)\cdot V_L}{Q_G(t)}\cdot C_{L,N2O}(t)-a_5(t)\cdot C_{G,N2O}(t) \qquad [A4.2]$$

in which

$$a_3(t)=\frac{k_La(t)\cdot Q_G(t)\cdot H}{Q_G(t)\cdot H+k_La(t)\cdot V_L} \qquad [A4.3]$$

and

$$a_5(t)=\frac{Q_G(t)}{V_G}=D_G(t) \qquad [A4.4]$$

Laplace transformation of the liquid and gas phase mass balances (Eqs. A4.1 and A4.2) yields, after rearrangement:

$$C_{L,N2O}(s)=\frac{1}{s+a_3(s)+D_L(s)}\cdot\left[R_{L,N2O}(s)+C_{L,N2O}(0)\right] \qquad [A4.5]$$

$$C_{G,N2O}(s)=\frac{1}{s+a_5(s)}\left[a_5(s)\cdot\frac{a_3(s)\cdot V_L}{Q_G(s)}\cdot C_{L,N2O}(s)+C_{G,N2O}(0)\right] \quad [A4.6]$$

In what follows, the gas and liquid flow rates Q_G and Q_L as well as the interphase transfer rate k_La are assumed constant, so a_3 and a_5 (Eqs. A4.3 and A4.4) are constant as well. This assumption is not limiting, since Q_G is constant for each aeration interval as such (high – low aeration intensity). Substitution of Eq. A4.5 in Eq. A4.6 then yields:

$$C_{G,N2O}(s)=\frac{1}{s+a_5}\left[a_5\cdot\frac{a_3\cdot V_L}{Q_G}\cdot\frac{1}{s+a_3+D_L}\cdot\left(R_{V,N2O}(s)+C_{L,N2O}(0)\right)+C_{G,N2O}(0)\right] \quad [A4.7]$$

Note that the volumetric N_2O formation rate $R_{V,N2O}(t)$ is not necessarily constant. Two different profiles for $R_{V,N2O}(t)$ are considered, being a constant and a linear profile.

Profile 1: constant N_2O formation rate

The solution of the mass balances Eqs. A4.5 and A4.6 for a constant N_2O formation rate $R_{V,N2O}(t)$ results in the following gas phase and liquid phase N_2O concentration profiles (Mampaey et al. 2015),

$$C_{G,N2O}(t)=a_1+a_2\cdot exp\left(-\left(a_3+D_L\right)\cdot t\right)-a_4\cdot exp\left(-a_5\cdot t\right) \quad [A4.8]$$

$$C_{L,N2O}(t)=a_1\cdot\frac{Q_G}{a_3\cdot V_L}+a_2\cdot\frac{a_5-\left(a_3-D_L\right)}{a_5}\cdot\frac{Q_G}{a_3\cdot V_L}\cdot exp\left(-\left(a_3+D_L\right)\cdot t\right) \quad [A4.9]$$

in which

$$a_1=\frac{a_3\cdot V_L}{Q_G}\cdot\frac{1}{a_3+D_L}\cdot R_{V,N2O} \quad [A4.10]$$

$$a_2=\frac{a_5}{a_5-\left(a_3+D_L\right)}\cdot\frac{a_3\cdot V_L}{Q_G}\cdot\left(C_{L,N2O}(0)-\frac{C_1}{a_3+D_L}\right) \quad [A4.11]$$

$$a_4=\frac{a_3\cdot V_L}{Q_G}\cdot\left(\frac{C_1}{a_3+D_L}+\frac{a_5}{a_5-\left(a_3+D_L\right)}\cdot\left(C_{L,N2O}(0)-\frac{C_1}{a_3+D_L}\right)\right)-C_{G,N2O}(0) \quad [A4.12]$$

The N_2O formation rate is obtained from Eq A4.10 as

$$R_{V,N2O} = a_1 \cdot \frac{a_3 + D_L}{a_3} \cdot \frac{Q_G}{V_L} \qquad \text{[A4.13]}$$

Profile 2: linear profile of N_2O formation rate in time

In case of a linear profile for the N_2O formation rate $R_{V,N2O}(t)$,

$$R_{V,N2O}(t) = C_1 \cdot t \qquad \text{[A4.14]}$$

with corresponding Laplace transform

$$R_{V,N2O}(s) = \frac{C_1}{s^2} \qquad \text{[A4.15]}$$

the measured gas concentration profile (solution of Eq. A4.7), after expansion in partial fractions, is given by Eq. A4.16:

$$\begin{aligned}
C_{G,N2O}(s) &= \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{1}{a_3 + D_L} \cdot \frac{C_1}{s^2} \\
&- \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{C_1}{a_3 + D_L} \cdot \left(\frac{1}{a_3 + D_L} + \frac{1}{a_5} \right) \cdot \frac{1}{s} \\
&+ \frac{a_5}{a_5 - (a_3 + D_L)} \cdot \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{C_1}{(a_3 + D_L)^2} \cdot \frac{1}{s + a_3 + D_L} \\
&+ \frac{a_5}{a_5 - (a_3 + D_L)} \cdot \frac{a_3 \cdot V_L}{Q_G} \cdot C_{L,N2O}(0) \cdot \frac{1}{s + a_3 + D_L} \\
&+ \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{C_1}{a_3 + D_L} \cdot \frac{1}{s + a_5} \\
&- \frac{a_5}{a_5 - (a_3 + D_L)} \cdot \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{C_1}{(a_3 + D_L)^2} \cdot \frac{1}{s + a_5} \\
&+ \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{C_1}{(a_3 + D_L)^2} \cdot \frac{1}{s + a_5} \\
&- \frac{a_5}{a_5 - (a_3 + D_L)} \cdot \frac{a_3 \cdot V_L}{Q_G} \cdot C_{L,N2O}(0) \cdot \frac{1}{s + a_5} \\
&+ C_{G,N2O}(0) \cdot \frac{1}{s + a_5}
\end{aligned} \qquad \text{[A4.16]}$$

The inverse Laplace transform yields, with substitution of $\frac{C_1}{s^2} = R_{V,N2O}(s)$:

$$C_{G,N2O}(t) = a_1(t) - a_6 + a_2 \cdot exp\left(-(a_3 + D_L) \cdot t\right) - a_4 \cdot exp(-a_5 \cdot t) \qquad \text{[A4.17]}$$

with time-varying coefficient

$$a_1(t) = \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{1}{a_3 + D_L} \cdot R_{V,N2O}(t) \qquad \text{[A4.18]}$$

and with

$$a_2' = \frac{a_5}{a_5 - (a_3 + D_L)} \cdot \frac{a_3 \cdot V_L}{Q_G} \cdot \left(C_{L,N2O}(0) + \frac{C_1}{(a_3 + D_L)^2} \right) \qquad \text{[A4.19]}$$

$$a_4' = \frac{a_3 \cdot V_L}{Q_G} \cdot \left(\frac{-C_1}{a_3 + D_L} - \frac{C_1}{(a_3 + D_L)^2} + \frac{a_5}{a_5 - (a_3 + D_L)} \cdot \left(C_{L,N2O}(0) + \frac{C_1}{(a_3 + D_L)^2} \right) \right) - C_{G,N2O}(0) \qquad \text{[A4.20]}$$

$$a_6 = \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{1}{a_3 + D_L} \cdot \left(\frac{C_1}{a_3 + D_L} + \frac{C_1}{a_5} \right) \qquad \text{[A4.21]}$$

In case the interphase transfer rate (proportionally related to a_3) and the headspace dilution rate (a_5) are high, the term a_6 can be neglected in Eq. A4.17, so the gas phase concentration profile in case of a linearly varying N_2O formation rate (Eq. A4.14) is described by:

$$C_{G,N2O}(t) = a_1(t) + a_2' \cdot exp(-(a_3 + D_L) \cdot t) - a_4' \cdot exp(-a_5 \cdot t) \qquad \text{[A4.22]}$$

which is the same expression as in case of a constant N_2O formation rate (Eq. A4.8), but now with a time-varying coefficient $a_1(t)$.

During the transient period, the gas phase N_2O concentration profile (Eq. A4.17 or Eq. 4.2) is dominated by the exponential functions, which are related to the initial reactor conditions. The reactor has attained **steady state** once the transients have vanished, which also implies that the term a_6 has become negligible compared to the exponential terms. Eq. 4.2 (or Eq. A4.17) is then reduced to Eq. A4.23.

$$C_{G,N2O}^{HighSS}(t) = a_1(t) \equiv \frac{a_3 \cdot V_L}{Q_G} \cdot \frac{1}{a_3 + D_L} \cdot R_{V,N2O}^{HighSS}(t) \qquad \text{[A4.23]}$$

which demonstrates that the steady state N_2O concentration profile is directly related to the N_2O formation rate $R_{V,N2O}(t)$. Rearrangement of Eq. A4.23 yields Eq. A4.24 (Eq. 4.5),

$$R_{V,N2O}^{HighSS}(t) = \frac{Q_G}{V_L} \cdot \frac{a_3}{a_3 + D_L} \cdot C_{G,N2O}^{HighSS}(t) \qquad [A4.24]$$

which can be applied to calculate the N_2O formation rate $R_{V,N2O}^{HighSS}(t)$ from the measured gas phase N_2O concentration profile $C_{G,N2O}^{HighSS}(t)$.

The liquid profile is obtained from inverse Laplace transformation of Eq. A4.5, after substitution of $R_{V,N2O}(t)$, Eq. A4.15, as:

$$C_{L,N2O}(t) = a_1(t) \cdot \frac{Q_G}{a_3 \cdot V_L} - \frac{a_1(0)}{a_3 + D_L} \cdot \frac{Q_G}{a_3 \cdot V_L} + a_2' \cdot \frac{a_5 - (a_3 - D_L)}{a_5} \cdot \frac{Q_G}{a_3 \cdot V_L} \cdot exp\left(-(a_3 + D_L) \cdot t\right) \qquad [A4.25]$$

When the interphase transfer rate is high (a_3 is high), the term $-\frac{R_{V,N2O}(0)}{(a_3 + D_L)^2}$ can be disregarded, so Eq. A4.25 is simplified to

$$C_{L,N2O}(t) = a_1(t) \frac{Q_G}{a_3 \cdot V_L} + a_2' \cdot \frac{a_5 - (a_3 - D_L)}{a_5} \cdot \frac{Q_G}{a_3 \cdot V_L} \cdot exp\left(-(a_3 + D_L) \cdot t\right) \qquad [A4.26]$$

which is the same expression as obtained for a constant N_2O formation rate (Eq. A4.9).

Under steady state conditions, Eq. A4.26 is further reduced to

$$C_{L,N2O}^{HighSS}(t) = \frac{Q_G}{a_3 \cdot V_L} \cdot a_1(t) \equiv \frac{Q_G}{a_3 \cdot V_L} \cdot C_{G,N2O}^{HighSS}(t) \qquad [A4.27]$$

A4.2.2 Application to full-scale data

The calculation of the volumetric N_2O formation rate and the liquid phase N_2O concentration profile from the gas-phase measurements is illustrated in this section considering the data of the first high aeration period (Figure 4. 5). Figure A4. 2 displays these off-gas data expressed in ppm. Upon visual inspection, the data are divided into two parts, a transient part consisting of a

N_2O peak which levels off to a constant value (6:50 to 7:11), followed by a steady state part characterized by a linear decrease in the gas phase N_2O concentration (7:18 to 8:31).

Fitting Eq. 4.2, with time t ranging from 0 to 21 minutes, to the transient part of the gas measurements in the 'high aeration' period yields

$$C_{G,N2O}(t) = 306 + 2786 \cdot exp(-0.3217 \cdot t) - 2554 \cdot exp(-0.3699 \cdot t) \quad \text{[A4.28]}$$

which directly gives the values of a_i.

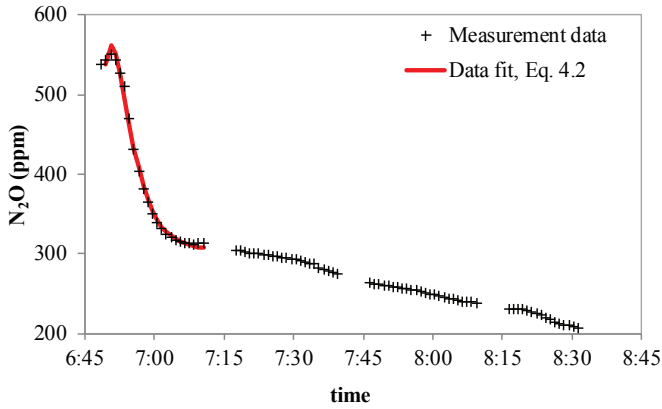

Figure A4. 2 Concentration profile of N_2O in the off-gas of the PNA granular sludge reactor and data fit of Eq. 4.2 during the transient period.

The N_2O formation rate $R_{V,N2O}$ during the transient period, which was assumed constant, is then directly calculated from Eq. 4.3 as

$$R_{V,N2O}^{HighT} = \frac{0.3217}{0.3217 - 0.0036} \cdot \frac{67.35}{600} \cdot 306 \cdot \left(10^{-6} \cdot \frac{101325}{8.3145 \cdot 310} \cdot 28\right) \cdot (60 \cdot 24) = 0.055 gN.m^{-3}.d^{-1} \quad \text{[A4.29]}$$

The factors between brackets denote the conversion of ppm to g N m^{-3}, and of min^{-1} to d^{-1}, respectively.

The dissolved N_2O concentration $C_{L,N2O}(t)$ during the transient period was calculated from Eq. 4.4 as

$$C_{L,N2O}^{HighT}(t)=\left(\frac{306\cdot 67.35}{0.3181\cdot 600}+2786\cdot\frac{0.3699-0.3217}{0.3699}\cdot\frac{67.35}{0.3181\cdot 600}exp(-0.3217\cdot t)\right)\cdot \left(10^{-6}\cdot\frac{101325}{8.3145\cdot 310}\cdot 28\right)=0.119+0.1408\cdot exp(-0.3217\cdot t) \quad [A4.30]$$

As for the transient period, fitting Eq. 4.2 to the measured gaseous N_2O profile yielded a R^2 value of 0.996.

During the steady state period, the N_2O formation rate and the dissolved N_2O concentration were directly calculated from the measured gas phase concentration through Eq. 4.6 and Eq. 4.7. The decrease in formation rate $R_{V,N2O}^{HighSS}$ caused the decreasing gas and liquid phase concentrations. Fitting a linear trendline yields a R^2 value of 0.993, also indicating a good fit during the steady state conditions.

The overall results for the N_2O formation rate and the dissolved N_2O concentration are shown in Figure A4. 3 and Figure A4. 4, respectively.

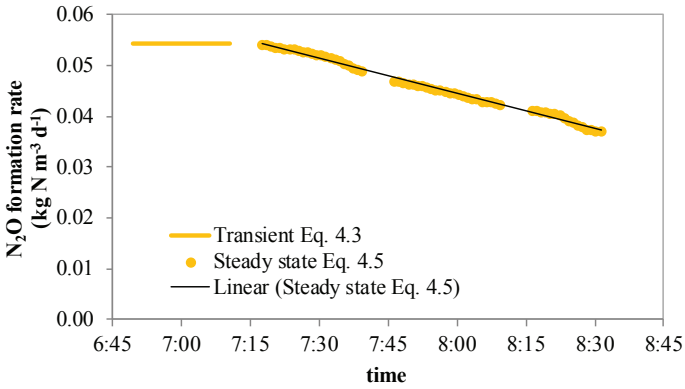

Figure A4. 3 Volumetric N_2O formation rate, calculated from Eq. 4. 3 during the transient period and from Eq. 4. 5 during steady state.

During the transient period, the dissolved N_2O concentration $C_{L,N2O}(t)$ was calculated from Eq. 4.4 during the transient period and from Eq. 4.6 during the steady state. The results are shown in Figure A4. 4.

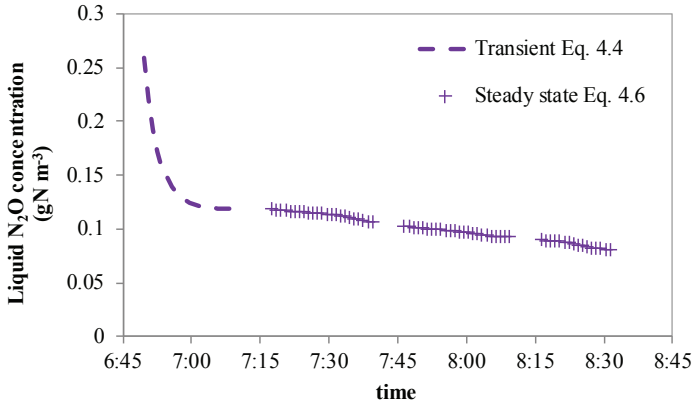

Figure A4. 4 Liquid N_2O concentration, calculated from Eq. 4.4 during the transient period and from Eq. 4.6 during steady state.

A4.3 COD removal during the N_2O monitoring campaign (August 22-29 2011)

The COD concentration in the inlet of the PHOSPAQ unit (phosphorus removal step) was calculated through mass balances, while the COD concentration in the PNA reactor was measured (see section 4.2.2 of the paper). Table A4. 1 summarizes these data and the corresponding COD removal efficiency over both phosphorus and nitrogen removal units for each day of the monitoring campaign.

Table A4. 1 COD concentration in the inlet of the PHOSPAQ unit, effluent of PNA reactor and COD removal efficiency in both units during the week of the N_2O monitoring campaign (August 22-29 August 2011).

Day	COD inlet PHOSPAQ (mg COD L^{-1})	COD in the PNA reactor (mg COD L^{-1})	COD removal (%)
22-08-11	608	190	69
23-08-11	532	190	64
24-08-11	528	190	64
25-08-11	527	160	70
26-08-11	536	160	70
27-08-11	660	160	76
28-08-11	664	170	74
29-08-11	568	170	70

A4.4 Ammonium oxidation rate (AOR)

The ammonium oxidation rate (AOR) was calculated for the selected interval to study the normal operation of the PNA reactor (Figure 4. 5) as well as for the dedicated experiment with the stop of the aeration followed by an enhanced aeration period (Figure 4. 6).

Mass balances for intervals of approximately 40 min were used for the calculation of the AOR, making use of the ammonium concentration in the effluent of the PNA reactor (online data), liquid flow rate (online data) and the ammonium concentration in the influent (340 mg L^{-1}, from a sample) ([A4.31]Eq. A4.31).

$$AOR = \left(C_{NH_4^+,INF} - C_{NH_4^+,EFF} \right) \cdot \frac{Q_L}{V_L} - \frac{\Delta C_{NH_4^+}}{\Delta t} \qquad [A4.31]$$

The results of the AOR calculation are showed in Figure A4. 5 for the normal operation of the reactor and in Figure A4. 6 for the dedicated experiment.

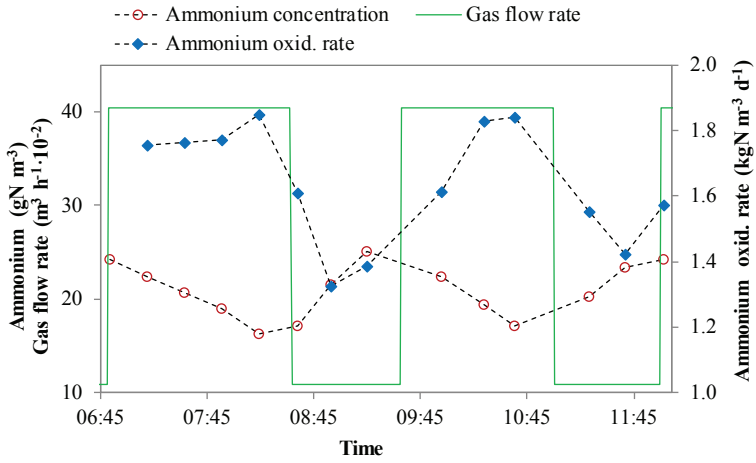

Figure A4. 5 Ammonium concentration and ammonium oxidation rate (AOR) calculated for the normal reactor operation of the PNA reactor on August 24th 2011.

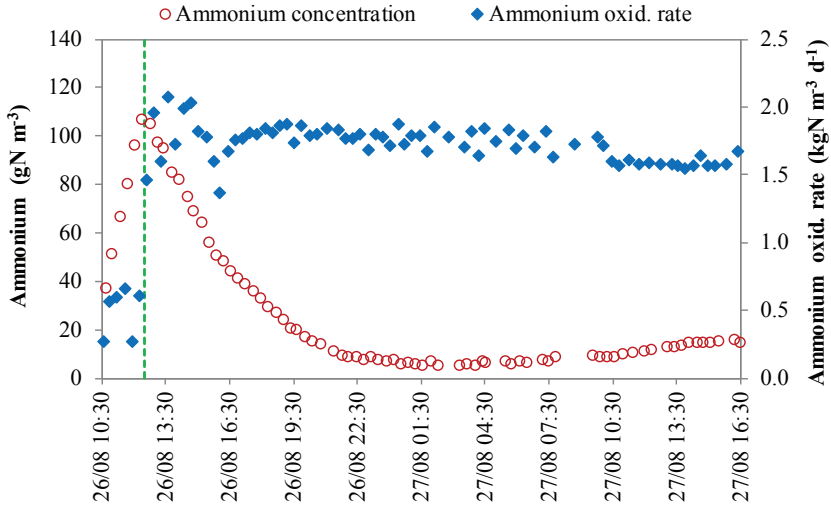

Figure A4. 6 Ammonium concentration and ammonium oxidation rate (AOR) calculated for the dedicated experiment with no aeration (10:30-12:30 on August 26th) and prolonged aeration (12:30 on 26/Aug to 16:30 on 27/Aug, after dotted line) carried out in the PNA reactor.

5

Integration of methane removal in aerobic anammox-based granular sludge reactors

Abstract

Apart from its high ammonium concentration, reject water from anaerobic digestion contains dissolved methane that can escape to the atmosphere, hence contributing to global warming. Ammonium removal from such concentrated streams through combined partial nitritation-anaerobic ammonium oxidation (anammox) processes has been widely studied and implemented in dedicated reactor types such as aerobic granular sludge reactors. This study investigates the possibility of integrating methane removal in these reactors, through modelling and simulation. Methane removal could be established through aerobic methane oxidizing bacteria (MOB), denitrifying anaerobic methane oxidizing bacteria (damoB, NO_2^- + CH_4 → N_2 + CO_2), and/or archaea (damoA, NO_3^- + CH_4 → NO_2^- + CO_2). The simulation results demonstrated that the combined removal of nitrogen and methane was feasible at low dissolved oxygen conditions (< 0.5 g O_2 m^{-3}). The nitrogen removal efficiency increased in the presence of anaerobic methanotrophic archaea, which reduce nitrate providing nitrite to anammox bacteria. Aerobic methane oxidizing bacteria were the main responsible microorganisms removing methane. A sensitivity analysis of key kinetic parameters influencing the coexistence of anaerobic (damoA and damoB) and aerobic methane oxidizers showed a shift of the methanotrophic populations depending on the most favourable parameters for each microbial group, while keeping high nitrogen and methane removal efficiencies. Possible methane stripping during aeration could be limited by increasing the depth within the reactor column at which the aeration was supplied. Overall, the integration of methane removal in aerobic anammox-based granular sludge reactors seems a promising process option to reduce the carbon footprint from wastewater treatment.

Chapter redrafted after:

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5.1 Introduction

Wastewater treatment negatively contributes to global warming by emitting the greenhouse gases carbon dioxide (CO_2), nitrous oxide (N_2O) (Kampschreur et al. 2009b) and methane (CH_4) (Daelman et al. 2012). CH_4 is a strong greenhouse gas that accounts for 34-CO_2 equivalents over a 100-year horizon (IPCC 2013). CH_4 can be emitted after its formation in the sewage system or can be stripped to the atmosphere from the reject water from the anaerobic digester. Even though the energy recovery of the dissolved CH_4 from the reject water may not be economically attractive, its removal could significantly decrease the carbon footprint of the wastewater treatment plant (WWTP) (Daelman et al. 2012).

Recently it was found that anaerobic CH_4 oxidation coupled to denitrification can take place (Raghoebarsing et al. 2006). Denitrifying anaerobic methane oxidation (damo) can be carried out by bacteria (damoB), namely *Candidatus* Methylomirabilis oxyfera, which couple CH_4 oxidation to nitrite reduction (Ettwig et al. 2010; Luesken et al. 2011) (Eq. 5.1) and by archaea (damoA) such as *Candidatus* Methanoperedens nitroreducens, that uses nitrate as electron acceptor (Haroon et al. 2013) (Eq. 5.2).

damoB $$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad [5.1]$$

damoA $$2CH_4 + 8NO_3^- \rightarrow 2CO_2 + 8NO_2^- + 4H_2O \quad [5.2]$$

Apart from its dissolved CH_4 content, reject water is in the first place characterized by high nitrogen concentrations in the form of ammonium and by high temperatures and is therefore suitable for the application of anaerobic ammonium oxidation (anammox) technology (Strous et al. 1998), implying important energy and chemical savings compared to conventional nitrogen removal over nitrate.

Simultaneous ammonium and CH_4 removal by anammox and damo microorganisms under anoxic conditions has been demonstrated feasible in lab-scale systems (Hu et al. 2015; Shi et al. 2013) and modelling approaches (Chen et al. 2014; Winkler et al. 2015). In practice, this system needs an additional step realizing nitritation (oxidation of ammonium to nitrite by ammonium oxidizing bacteria (AOB)) in order to achieve the complete

ammonium and CH_4 (when considering damoB, Eq. 5.1) removal. Ammonium can be conveniently removed from reject water with a combined partial nitritation-anammox process (Fux et al. 2002; van der Star et al. 2007), which can be implemented in one or two stages.

Simultaneous ammonium and CH_4 removal in aerobic reactors, by combining partial nitritation-anammox systems with damo conversions, so far has not been realized experimentally. Chen et al. (2015) addressed this topic in a modelling study, considering a membrane biofilm reactor of 1 m^3 to supply oxygen while avoiding CH_4 stripping. They assumed that aerobic methane oxidizers (MOB) would not be present in the system since oxygen and CH_4 diffused from opposite sides in the biofilm. Given that membrane systems are costly and prone to fouling, the present study assessed the potential integration of CH_4 conversion in aerated partial nitritation-anammox granular sludge reactors, reducing CH_4 stripping by keeping the aeration minimal.

The presented model is the first to include not only damo bacteria and archaea, but also MOB as a potentially important microbial group for CH_4 removal, besides the bacterial populations playing a role in nitrogen removal (AOB, NOB, anammox bacteria) and heterotrophs (growing from decay products), in a one stage aerobic anamox-based granular sludge reactor. Microbial competition in the granules was investigated through simulation, determining the key parameters that govern the presence or absence of the different populations. A sensitivity analysis was carried out to assess the influence of the microbial parameter values in this respect and the potential minimization of CH_4 stripping through adequate reactor design was assessed.

5.2 Materials and methods

5.2.1 Modelling simultaneous nitrogen and methane removal

A model was set up to describe growth and decay of damo bacteria (damoB), damo archaea (damoA), anammox bacteria, ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), aerobic methane oxidizing bacteria

(MOB), and heterotrophic bacteria. Nitrification and anammox reactions were modelled based on Volcke et al. (2010), the heterotrophic transformations were modelled according to Henze et al. (2000) and the aerobic methane oxidation was based on Arcangeli and Arvin (1999). The damoB process was modelled according to Winkler et al. (2015), and in addition the growth and decay of damoA (besides other species) was added in this study. The interactions between all microbial groups involved are summarized in Table 5. 1.

Table 5. 1 Overview of microbial competition for substrates (S) and dependencies through formed products (P) during simultaneous nitrogen and CH_4 removal in aerobic reactors.

	NH_4^+	NO_2^-	NO_3^-	O_2	N_2	CH_4
Aerobic populations						
AOB	S	P		S		
NOB		S	P	S		
MOB				S		S
Aerobic heterotrophs				S		
Anaerobic populations						
Anammox bacteria	S	S	P		P	
damoB		S			P	S
damoA		P	S			S
Anaerobic het. (NO_2^-)		S			P	
Anaerobic het. (NO_3^-)		P	S			

The kinetic expressions and stoichiometric matrix concerning the methanotrophic populations are summarized in Table 5. 2 and 5. 3. The corresponding parameter values for the methanotrophs and anammox bacteria are presented in Table 5. 4. Note that damoB could use both nitrite (S_{NO2}) and ammonium (S_{NH}) as nitrogen source to grow. Since nitrite is involved in the catabolic metabolism of damoB, this substrate needs to be present for the survival of the bacteria. Therefore, nitrite was considered the nitrogen source for damoB in this study, as in the model of Winkler et al. (2015) and in accordance with the findings of Ettwig et al. (2008). The oxygen inhibition constants for damoB and damoA ($K_{iO2\_damoB/A}$) are not available from literature and were considered to be the same as for anammox bacteria, 0.01 g O_2 m^{-3} (Hao et al. 2002). The nitrite inhibition constant (K_{iNO2_damoA}) and

CH_4 half saturation constant (K_{CH4_damoA}) for damoA were assumed the same as for damoB, 40 g N m^{-3} and 0.19 g COD m^{-3}, respectively.

The detailed model kinetics and stoichiometry for all reactions (including nitrogen conversions) are available in the Appendix A5.1.

Table 5. 2 Kinetic expressions for the methanotrophic microorganisms involved in the model.

1	Growth of damoB	$\mu_{max}^{damoB} \cdot \frac{S_{CH4}}{K_{CH4}^{damoB} + S_{CH4}} \cdot \frac{S_{NO2}}{K_{NO2}^{damoB} + S_{NO2}} \cdot \frac{K_{i-NO2}^{damoB}}{K_{i-NO2}^{damoB} + S_{NO2}} \cdot \frac{K_{i-O2}^{damoB}}{K_{i-O2}^{damoB} + S_{O2}} \cdot X_{damoB}$
2	Decay of damoB	$b^{damoB} \cdot X_{damoB}$
3	Growth of damoA	$\mu_{max}^{damoA} \cdot \frac{S_{NH}}{K_{NH}^{damoA} + S_{NH}} \cdot \frac{S_{CH4}}{K_{CH4}^{damoA} + S_{CH4}} \cdot \frac{S_{NO2}}{K_{NO2}^{damoA} + S_{NO2}} \cdot \frac{K_{i-NO2}^{damoA}}{K_{i-NO2}^{damoA} + S_{NO2}} \cdot \frac{K_{i-O2}^{damoA}}{K_{i-O2}^{damoA} + S_{O2}} \cdot X_{damoA}$
4	Decay of damoA	$b^{damoB} \cdot X_{damoB}$
5	Growth of MOB	$\mu_{max}^{MOB} \cdot \frac{S_{NH}}{K_{NH}^{MOB} + S_{NH}} \cdot \frac{S_{O2}}{K_{O2}^{MOB} + S_{O2}} \cdot \frac{S_{CH4}}{K_{CH4}^{MOB} + S_{CH4}} \cdot X_{MOB}$
6	Decay of MOB	$b^{MOB} \cdot X_{MOB}$

Table 5. 3 Stoichiometric matrix for the methanotrophic microorganisms involved in the model.

Comp. →	S_{NH}	S_{NO2}	S_{NO3}	S_{N2}	S_{O2}	S_{CH4}	S_S	X_{damoB}	X_{damoA}	X_{MOB}	X_I
Process ↓	g N m^{-3}				g O_2 m^{-3}	g COD m^{-3}					
Damo bacteria (damoB)											
1		$-\frac{1}{Y_{damoB}} + i_{NXB}$		$\frac{1}{Y_{damoB}} - 2i_{NXB}$		$-\frac{1.71}{Y_{damoB}} - 1$		1			
2	$i_{NXB} - f_I i_{NXI} - (1 - f_I) i_{NSS}$						$1 - f_I$	−1			f_I
Damo archaea (damoA)											
3	$-i_{NXB}$	$\frac{1}{1.14}\left(\frac{1}{Y_{damoA}} - 1\right)$	$-\frac{1}{1.14}\left(\frac{1}{Y_{damoA}} - 1\right)$			$-\frac{1}{Y_{damoA}}$			1		
4	$i_{NXB} - f_I i_{NXI} - (1 - f_I) i_{NSS}$						$1 - f_I$		−1		f_I
Aerobic methane oxidizing bacteria (MOB)											
5	$-i_{NXB}$				$1 - \frac{1}{Y_{MOB}}$	$-\frac{1}{Y_{MOB}}$				1	
6	$i_{NXB} - f_I i_{NXI} - (1 - f_I) i_{NSS}$						$1 - f_I$			−1	f_I

Table 5. 4 Kinetic and stoichiometric parameters for methanotrophs and anammox bacteria.

	Description	Anammox bacteria	DamoB	DamoA	MOB	Unit
Y_X	Yield on ammonium	0.170 (Strous et al. 1998)	0.156* (Winkler et al. 2015)	0.071 (Rasigraf et al. 2012)	0.2 (Arcangeli and Arvin 1999)	g COD g N^{-1}
μ_{max}^X	Maximum growth rate	0.052 (Strous et al. 1998)	0.0495* (Ettwig et al. 2010)	0.0362 (Chen et al. 2014)	3.5 (Arcangeli and Arvin 1999)	d^{-1}
K_{NH4}^X	Ammonium half saturation constant	0.037 (Hao et al. 2002)	-	0.02 Assumed	2 (Arcangeli and Arvin 1997)	g N m^{-3}
K_{NO2}^X	Nitrite half saturation constant	0.005 (Hao et al. 2002)	0.6 (He et al. 2013)	-	-	g N m^{-3}
K_{NO3}^X	Nitrate half saturation constant	-	-	0.11 Chen et al. (2014)	-	g N m^{-3}
K_{CH4}^X	Methane half saturation constant	-	0.19 (Raghoebarsing et al. 2006; Ettwig et al. 2008)	0.19 Assumed same as damoB	0.26 (Arcangeli and Arvin 1999)	g COD m^{-3}
K_{O2}^X	Oxygen half saturation constant	-	-	-	0.2 (Arcangeli and Arvin 1997)	g O_2 m^{-3}
K_{i-NO2}^X	Nitrite inhibition constant	400 (Lotti et al. 2012)	40 (He et al., 2013; Rasigraf et al., 2012)	40 Assumed same as damoB	-	g N m^{-3}
K_{i-O2}^X	Oxygen inhibition constant	0.01 (Hao et al. 2002)	0.01 Assumed same as AN	0.01 Assumed same as AN	-	g O_2 m^{-3}
b^X	Specific biomass decay rate	0.0026 Assumed 0.05 μ_{max}^{AN}	0.0025 Assumed 0.05 μ_{max}^{damoB}	0.0018 Assumed 0.05 μ_{max}^{damoA}	0.175 Assumed 0.05 μ_{max}^{MOB}	d^{-1}

* Yield and maximum growth rate for damoB on nitrite

5.2.2 Granular sludge reactor model

The abovementioned bioconversion reactions were implemented in a one-dimensional biofilm model in the Aquasim simulation environment (Reichert 1994). A biofilm compartment was used, considering spherical biomass particles (granules) with a fixed granule size of 0.75 mm. The reactor volume was 400 m^3 of which 100 m^3 were occupied by particulate material, comprising both active biomass as well as inerts generated during the decay, and the remaining volume (300 m^3) was occupied by the bulk liquid.

The interphase (gas-liquid) transfer rate for CH_4 and oxygen (TR_i) was included in the model to assess CH_4 stripping in the system (section 5.3.5). The partial pressure of the dissolved gases was modelled as a function of the reactor height as in Daelman et al. (2014) (see Appendix, section A5.5).

5.2.3 Set-up of the simulation study

The reactor behaviour was simulated at a liquid flow rate of 1000 $m^3\ d^{-1}$ and fixed CH_4 influent concentration of 100 g COD m^{-3} (25 g CH_4 m^{-3}) as in Winkler et al. (2015) and Chen et al. (2015). The influent was assumed to not contain any other organic carbon source, which is a reasonable assumption for reject water (Hao et al. 2002) and allows straightforward interpretation of the simulation results. Ammonium was the sole source of nitrogen in the influent and its concentration was varied from 100 to 1500 g N m^{-3}, covering low nitrogen concentrations for reject water (Henze et al. 2008) and the range for reject water in systems performing partial nitritation-anammox (Lackner et al. 2014). A sufficiently long simulation time was applied to reach steady state in terms of both bulk liquid concentrations and solid concentration profiles within the granules. An overview of the different simulations carried out and the specific settings for each case is provided in Table 5. 5.

While all bioconversions (Appendix, Table A5. 1) were included in most simulations, some simulations were run without damoA (i.e. by excluding reaction 3 and 4 from Table 5. 2) to assess their effect on the nitrogen removal.

Table 5. 5 Overview of simulation runs performed.

	Section	Influent ammonium concentration (g N m^{-3}) 100 300 500 700 900 1100 1300 1500	Kinetic parameters	Populations	Oxygen concentration
Influence of influent ammonium and bulk oxygen concentrations	5.3.1 5.3.2 5.3.3		Table A5. 3	All	Fixed (0.1 - 2.0 g O_2 m^{-3})
Influence of damo on N removal	5.3.2		Table A5. 3	Without damoA and/ or damoB	Fixed (0.1 - 0.2 g O_2 m^{-3})
Sensitivity analysis half saturation constants	5.3.4		K_{CH4_damo} changing (Table 5. 5)	All	Fixed (0.2 g O_2 m^{-3})
			K_{NH4_MOB} changing (Table 5. 5)	All	Fixed (0.2 g O_2 m^{-3})
			K_{NO2_damoB} changing (Table 5. 5)	All	Fixed (0.2 g O_2 m^{-3})
CH_4 stripping evaluation	5.3.5		Table A5. 3	All	Dependent on aeration (kLa O_2 = 0-600 d^{-1}) ≡ (0-0.4 g O_2 m^{-3})

Due to the significant differences found in literature for the half saturation constants of damoA, damoB and MOB, a sensitivity analysis was performed to determine their influence on the competition among methanotrophic communities (Table 5. 6).

Table 5. 6 Range of half saturation constants evaluated in this study.

Parameter	Value	Unit	Reference	Evaluation in this study
K_{CH4_damo}	5.888	g COD m^{-3}	(Chen et al. 2015)	0.1-8.0
	0.038	g COD m^{-3}	(Raghoebarsing et al. 2006)	
	0.32	g COD m^{-3}	(Ettwig et al. 2008)	
K_{NH4_MOB}	2	g N m^{-3}	(Arcangeli & Arvin 1997)	0.5-2.5
	≤ 1	g N m^{-3}	(Henze et al., 2000)	
K_{NO2_damoB}	0.01	g N m^{-3}	(Chen et al. 2015)	0.002-1.0
	0.6	g N m^{-3}	(Winkler et al. 2015)	

Gas-liquid interphase transport was neglected in most simulations, corresponding with an ideal scenario without CH_4 stripping, to assess the maximum CH_4 conversion potential. The oxygen concentration was then set at a fixed value between 0.1 and 2.0 g O_2 m^{-3} (with an intermediate spacing of 0.1 g O_2 m^{-3}), reflecting ideal oxygen control at a constant set-point. Besides, a series of simulations were carried out to assess the extent of CH_4 stripping and its potential mitigation by adding the aeration at different depths in the reactor (Appendix, section A5.5).

5.3 Results and discussion

5.3.1 Maximum nitrogen and methane removal efficiencies in aerobic granular sludge reactors

The maximum nitrogen and CH_4 removal efficiencies achieved as a function of the influent ammonium concentration, as well as the corresponding dissolved oxygen (DO) concentration ranges, are summarized in Figure 5. 1 (see Appendix, section A5.3 for more simulation results). Nitrogen removal efficiencies up to 99% were obtained for influent ammonium concentrations up to 1500 g N m^{-3}, when applying low optimal oxygen concentrations (< 0.5 g O_2 m^{-3}). At the highest applied influent ammonium concentration, the

nitrogen removal efficiency decreased significantly (down to 52.6% for influent ammonium concentration of 1500 g N m^{-3}) (Figure 5. 1) because the corresponding ammonium loading rate exceeded the biomass specific removal capacity (under dimensioning of the reactor). The fluctuation in the nitrogen removal efficiency for influent ammonium concentrations up to 1500 g N m^{-3} (Figure 5. 1) may be explained through its high sensitivity to the DO concentration. Nitrogen removal efficiency varied around 30.7 ± 15.7% when changing the DO ± 0.1 g O_2 m^{-3} (Appendix, Figure A5. 2). The sensitivity of the nitrogen removal efficiency to the DO concentration was observed also in an aerobic anammox-based granular sludge reactor without CH_4 removal (Volcke et al., 2010). Note that a broader range of optimal oxygen concentrations corresponding with maximum nitrogen removal was obtained in the presence of organic substrate in the influent due to the conversion nitrate to nitrite by heterotrophic communities (Mozumder et al., 2014).

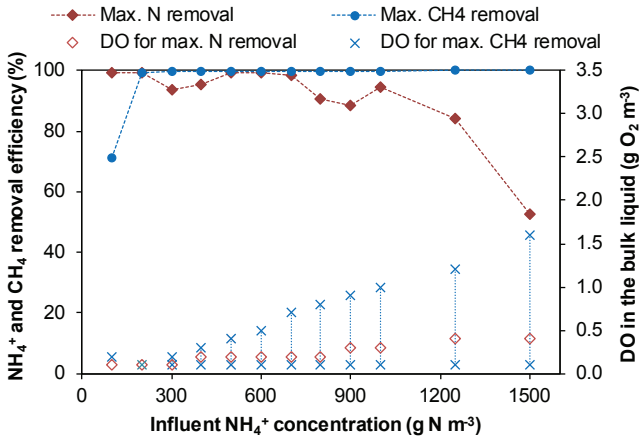

Figure 5. 1 Maximum nitrogen and CH_4 removal efficiencies and corresponding DO concentration (range) in terms of influent ammonium concentration. Simulation results obtained for ideal scenario without CH_4 stripping.

Almost complete (≥ 99%) CH_4 removal was found for influent ammonium concentrations of 200 g N m^{-3} or higher (Figure 5. 1). The corresponding optimal DO concentration interval giving high CH_4 removal efficiencies became broader with increasing influent ammonium concentration (Figure 5. 1). Interestingly, this DO concentration interval always included the DO for maximum nitrogen removal. Thus, maximum CH_4 removal can be achieved

under the same (DO) conditions that lead to maximum nitrogen removal. Only in case of a low influent ammonium concentration (NH_4^+ = 100 g N m^{-3}, keeping a constant CH_4 concentration), the maximum CH_4 removal was only 71% and required a slightly higher DO concentration (DO = 0.2 g O_2 m^{-3}) than the one corresponding with maximum nitrogen removal (DO = 0.1 g O_2 m^{-3}). The maximum CH_4 removal efficiencies found in the present study (≥ 99%), without considering CH_4 stripping, are higher than those obtained in the simulation study of Chen et al. (2015) (93%), where a membrane biofilm bioreactor was used and CH_4 stripping was avoided by supplying oxygen through the membrane, despite the higher CH_4 surface loading rate applied in this study (0.25 g COD m^{-2} d^{-1} in this study and 0.05-0.1 g COD m^{-2} d^{-1} in Chen et al. (2015)). The difference in CH_4 removal obtained in both studies could be related to the MOB activity, which was not included in the model of Chen et al. (2015).

5.3.2 Contribution of functional groups to CH_4 and nitrogen removal

Considering the cases with maximum nitrogen removal (Figure 5. 1), MOB were the main contributors to the CH_4 removal in most cases. Over >58% of CH_4 was removed by MOB, except for influent ammonium of 100 g N m^{-3}, with no MOB contribution on the CH_4 removal; and ammonium of 400 g N m^{-3}, with 18% CH_4 removed by MOB (Figure 5. 2). For influent ammonium concentrations higher than 800 g N m^{-3}, the nitrogen loading exceeded the biomass removal capacity leading to high nitrite accumulation (Appendix, Figure A5. 1), inhibiting damoA and leaving MOB as the only contributor for CH_4 removal. Between 400 and 800 g N m^{-3}, accumulation of nitrite only took place at DO concentrations higher than the optimal one corresponding with the maximum nitrogen removal efficiency (≤0.2 g O_2 m^{-3}), resulting in relatively important (28-41%) participation of damoA on CH_4 removal for influent ammonium concentrations in the range 600-800 g N m^{-3}.

With respect to the overall sustainability of the process, it can be noted that, even though the aeration energy associated with oxygen supply to aerobic methane oxidizers entails CO_2 emissions, this contribution to the carbon

footprint is significantly lower than the carbon footprint related to the direct emission of CH_4 (simple calculation in the Appendix, A5. 5).

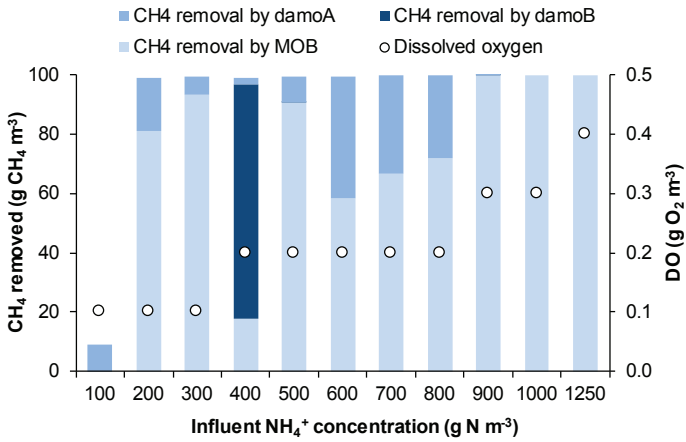

Figure 5. 2 CH_4 removed by each methanotrophic organism (influent CH_4 concentration = 100 g COD m^{-3}) in terms of the influent ammonium concentration, for the scenarios with maximum nitrogen removal efficiency.

For an influent ammonium concentration of 100 g N m^{-3}, damoA were the only microorganisms removing CH_4 (9.1% removal efficiency). DamoB and MOB did not survive in this case due to the limiting substrate availability (nitrite for damoB and oxygen and ammonium for MOB). DamoB only participated in the CH_4 removal for the maximum nitrogen removal scenarios when the influent ammonium concentration was 400 g NH_4^+-N m^{-3} (Figure 5. 2). In this case, AOB produced more nitrite than required by anammox bacteria to convert the remaining ammonium, resulting in a slight accumulation of nitrite in the system (9.6 g N m^{-3}, Appendix, Figure A5. 1C), hence creating a competitive edge for damoB (note that damoB have a lower affinity for nitrite than anammox bacteria, Table 5. 4). This conclusion is supported by a simplified calculation based on the ammonium consumed by AOB is presented in the Appendix (section A5.4).

Regarding the contribution of microbial groups to nitrogen removal, most of the nitrogen gas was produced by anammox bacteria (Appendix, Figure A5. 3). The heterotrophs (growing on decay products) had a small participation in the direct nitrogen formation with a maximum production of 3.4% of the

total nitrogen formed for the scenario with influent ammonium of 400 g N m^{-3}. Note that heterotrophic bacteria could also contribute to nitrogen formation indirectly by reducing nitrate to nitrite, which could be then further converted to nitrogen gas by anammox bacteria (see Mozumder et al. (2014)). Besides, this scenario was the only one where damoB had a relatively significant contribution (11%) to the nitrogen production, still being 8 times lower than the contribution by anammox bacteria (86% of the nitrogen produced).

The damo process does not only involve CH_4 removal, but also contributes to nitrogen removal, either directly through the conversion of nitrite to nitrogen gas by damoB or indirectly through the conversion by damoA of nitrate to nitrite, which can further be taken up by anammox or heterotrophic bacteria. The specific contribution of the damo process to the nitrogen removal in the system was studied for those optimal scenarios (maximum nitrogen and CH_4 removal according to Figure 5. 1) in which damoA and/or damoB were present. For these influent ammonium concentrations (from 100 to 800 g N m^{-3}, see Figure 5. 3), simulations were carried out without including damoA and/or damoB in the model (Figure 5. 3). For influent ammonium of 100, 200 and 500 g N m^{-3}, the presence of damoA did not influence the nitrogen removal. For these case scenarios, when the activity of damoA was not included in the model, the conversion of nitrate to nitrite by heterotrophs was the same as in the system considering damoA, and thus the global nitrogen removal was the same (the nitrite obtained either by damoA or heterotrophs was taken up by anammox bacteria yielding nitrogen gas) (Appendix, Figure A5. 4). At 300 g NH_4^+-N m^{-3}, the presence of damoA led to a slightly higher nitrogen removal efficiency. The activity of damoA at 400 g NH_4^+-N m^{-3} was little (1.9% CH_4 removed by damoA, Figure 5. 2), not influencing significantly the nitrogen removal. For this specific case (400 g NH_4^+-N m^{-3}), the presence of damoB decreased the nitrogen removal in the system from 98% (no damo processes considered) to 95% (damoB + damoA included or only damoB, since the activity of damoA was negligible) (Figure 5. 3). As mentioned before, damoB participated in the CH_4 removal when nitrite was available (slightly accumulation), which explains the that the overall nitrogen removal was not improved with the presence of damoB.

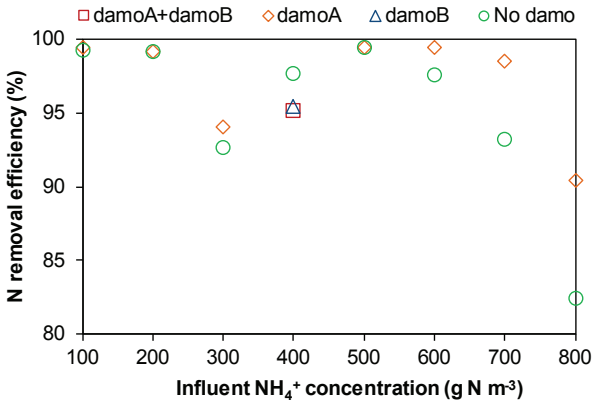

Figure 5. 3 Total N removal efficiency at different influent ammonium concentrations obtained when considering both damoB and damoA (□), only damoA (◊), only damoB (Δ) and no damo processes (○) in the model. Bulk oxygen concentration 0.1 g O_2 m^{-3} for 100-300 g NH_4^+-N m^{-3} and 0.2 g O_2 m^{-3} for 400-800 g NH_4^+-N m^{-3}.

At relatively high influent ammonium concentrations (600-800 g NH_4^+-N m^{-3}), damoA were present and increased the nitrogen removal efficiency (Figure 5. 3) through their synergetic interaction with anammox bacteria: nitrate produced by anammox bacteria was converted by damoA to nitrite, which was then again available for anammox bacteria, leading to higher nitrogen removal efficiencies. The positive effect of damoA on anammox conversion is comparable to the influence of heterotrophic bacteria, which may also improve the nitrogen formation by anammox through the conversion of nitrate to nitrite (Mozumder et al. 2014). In this study, the contribution of damoA to the conversion of nitrate to nitrite is higher than for heterotrophs, since the influent does not contain organic matter, so the growth of heterotrophs can only take place on decay products released. Overall, the presence of damoA positively influences the total nitrogen removal efficiency, while damoB activity does not due to the need to have nitrite available in excess to allow these bacteria to survive.

5.3.3 Microbial distribution

The microbial distribution in the granular sludge system for the scenarios with maximum nitrogen removal efficiencies (Figure 5. 1) is displayed in Figure 5. 4 (fraction of each population) and Figure 5. 5 (relative distribution

inside the granules). AOB and Anammox bacteria dominated the granules while the fraction of methanotrophs (damoA, damoB and/or MOB) was low (Figure 5. 4), corresponding with the relatively high NH_4^+ load compared to the CH_4 load. NOB were not present in the system at the limited DO concentrations required for maximum nitrogen and CH_4 removal.

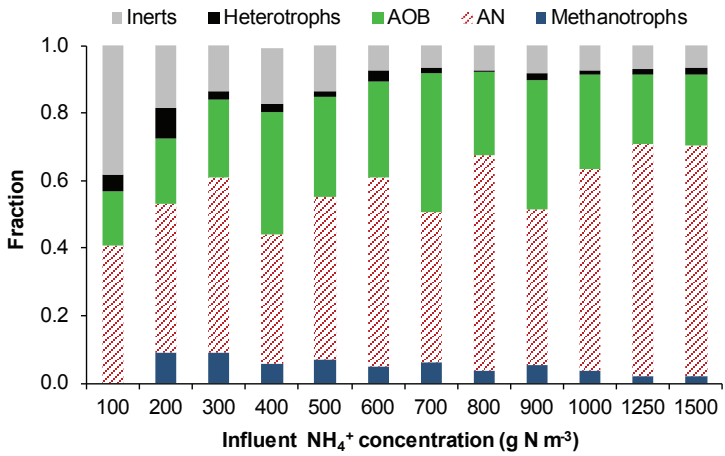

Figure 5. 4 Fraction of biomass and particulate inerts in terms of the influent ammonium concentrations, for the maximum N removal scenario (see Figure 5. 1).

AOB and aerobic MOB governed the aerobic outer part of the granules (Figure 5. 5). Anammox bacteria and damoA shared space inside the granule and for the specific case where damoB were present (Figure 5. 5A), these bacteria were located in the inner part of the anoxic zone. The relative location of anammox bacteria and damoB is in accordance with the findings of Winkler et al. (2015), for an anoxic (non-aerated) granular sludge system.

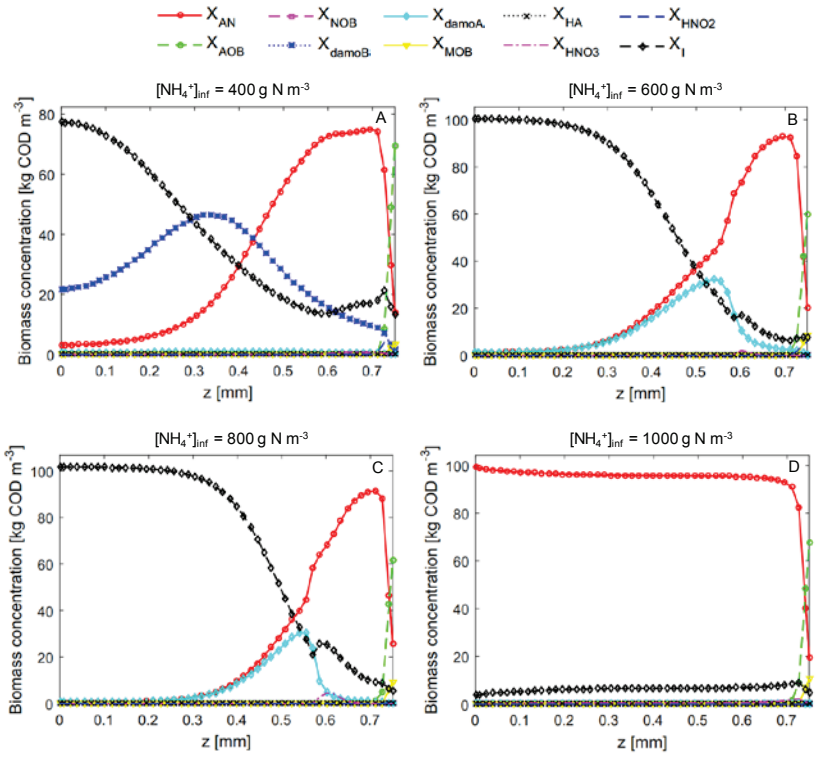

Figure 5. 5 Microbial distribution inside the granules. Axis x represents the radius of the granule, from 0 (the innermost part of the granule) to 0.75 mm (outer part of the granule).

The coexistence graph (Figure 5. 6) summarizes the presence of the methanotrophic groups (damoA, damoB and MOB) in the system for a range of influent ammonium and bulk oxygen concentrations. The regions between thick black lines correspond to the scenarios with maximum nitrogen removal. DamoA or damoA + damoB (regions of A and A+B in Figure 5. 6) were favoured at relatively low ammonium concentrations where the conversion of ammonium was high (see cases in Figure A5. 1 at DO > 0.1 g O_2 m^{-3}). In these cases where ammonium was not in excess, the low affinity of MOB for ammonium (nitrogen source for their growth) impeded their growth. Since MOB growth was limited, the CH_4 affinity played a more important role and thus damoA and damoB had advantage over MOB (K_{CH4_damo} = 0.19 g COD m^{-3} and K_{CH4_MOB} = 0.26 g COD m^{-3}, Table 5. 4). However, when sufficient

ammonium was available as nitrogen source for biomass growth, MOB were competitive because of their high growth rate (μ_{max_MOB} = 3.5 d^{-1}, μ_{max_damoA} = 0.036 d^{-1}, μ_{max_damoB} = 0.050 d^{-1}, Table 5. 4).

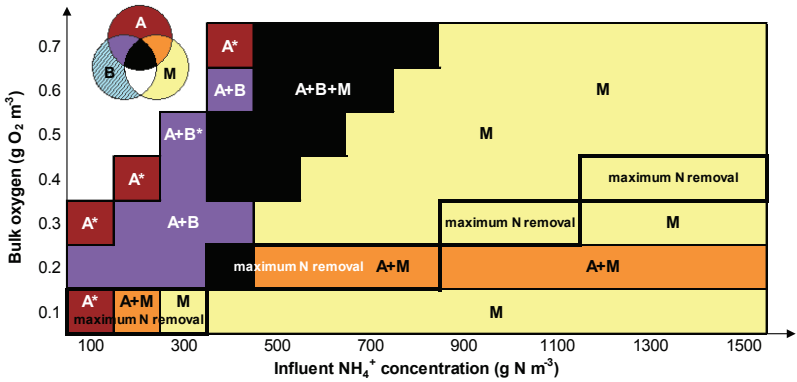

Figure 5. 6 Coexistence of methane oxidizers in terms of influent ammonium and DO concentrations for regions with significant (10-50%, see Figure A5. 2) CH_4 removal efficiency. The section between thick black lines corresponds to optimal nitrogen removal (see Figure 5. 1). The total methanotrophic population made up 1 to 10% of the total particulate fraction, except for regions marked with *, where the methanotrophs represented less than 1%. Methanotrophic populations were included when their presence was ≥ 0.1% of the methanotrophic community. M = MOB; A = damoA; B = damoB.

DamoA coexisted with MOB in all cases except when there was a high accumulation of nitrite, causing their inhibition (K_{iNO2_damoA} = 40 g NO_2^--N m^{-3}). This is the reason why at high ammonium concentrations and DO ≠ 0.2 g O_2 m^{-3} only MOB existed but not damoA (no accumulation of nitrite at 0.2 g O_2 m^{-3}, see Figure A5. 1). Apart from the methanotrophic competition, damoB compete also with anammox bacteria for nitrite. In this respect, damoB only survived when nitrite was not limited in the system. DamoB are the least favoured anaerobic methane oxidizers, and never appeared alone, but always with damoA or damoA + MOB (Figure 5. 6). The presence of damoB besides damoA and MOB took place when the ammonium was not in excess and at relatively high DO concentrations. An exception is the case for influent ammonium of 400 g N m^{-3} and relatively low DO = 0.2 g O_2 m^{-3}, in which MOB were present but damoB were the main contributor to CH_4 removal (80% CH_4 removed by damoB, Figure 5. 2). In this case, MOB still survived at the low DO concentration of 0.1 g O_2 m^{-3} due to their higher or equal affinity for oxygen

than the other aerobic populations (K_{O2_MOB} = 0.2 g O_2 m^{-3}; K_{O2_AOB} = 0.3 g O_2 m^{-3}; and K_{O2_HA} = 0.2 g O_2 m^{-3}) and their high growth rate.

In general, not all methanotrophic groups were present under the maximum CH_4 removal conditions (only one case, 400 g NH_4^+-N m^{-3}, Figure 5. 5 and Figure 5. 6).

5.3.4 Sensitivity analysis for the coexistence of the methanotrophic communities and substrate removal

A sensitivity analysis was performed to assess the influence of the methane half saturation constant for damoA and damoB (K_{CH4_damo}), the ammonium half saturation constant for MOB (K_{NH4_MOB}) and nitrite half saturation constant for damoB (K_{NO2_damoB}) on the presence of the methanotrophs and on the reactor performance in terms of nitrogen and CH_4 removal efficiencies (Table 5. 6). The analysis was performed for the scenario with maximum nitrogen and CH_4 removal in which the three methanotrophic groups (damoA, damoB and MOB) were present, i.e. for influent ammonium = 400 g N m^{-3} and DO = 0.2 g O_2 m^{-3} (see Figure 5. 1 and Figure 5. 6).

Decreasing the methane affinity of damo species (i.e. increasing K_{CH4_damo}) led to their outcompetition by MOB, which was complete for $K_{CH4\_damo} \geq 1$ g COD m^{-3} (Figure 5. 7A). The K_{CH4_damo} for damoB and damoA in the present study is 30 times lower (higher affinity) than in Chen et al. (2015) (K_{CH4_damo} = 0.19 g COD m^{-3} versus 5.888 g COD m^{-3}, respectively). While Chen et al. (2015) did not include MOB in the model to describe their system, our results indicate that MOB could have played an important role in their case.

Lower values for the ammonium half saturation constant of MOB (K_{NH4_MOB}) than the one used in this study (2 g COD m^{-3}) led to the dominance of MOB among methanotrophs (Figure 5. 7B). Even though ammonium only serves as a nitrogen source for biomass growth of MOB, the change in K_{NH4_MOB} plays an important role, especially at low ammonium concentrations, which leads to their growth limitation.

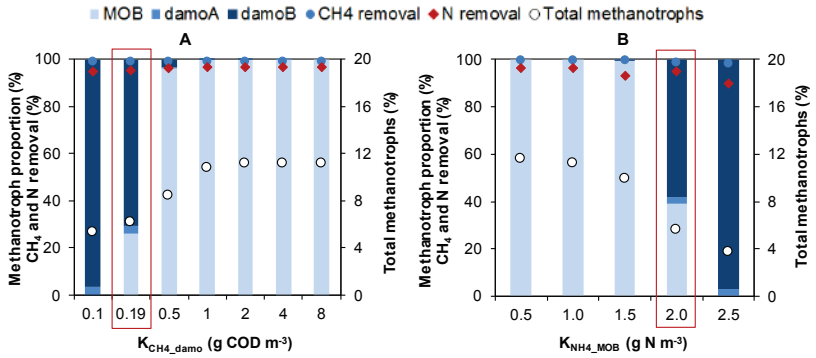

Figure 5. 7 Influence of A) methane half saturation constant for damo organisms and B) ammonium half saturation constant for MOB on the total fraction of methanotrophs among all particulate components, relative fraction of methanotrophic microorganisms within the total methanotrophs and, nitrogen and CH_4 removal efficiencies. Influent NH_4^+ = 400 g N m^{-3}; DO = 0.2 g O_2 m^{-3}. The box indicates the case with standard parameter values applied in this study.

Decreasing the nitrite half saturation constant for damoB (K_{NO2_damoB}) (hence increasing competitiveness for nitrite) resulted in increasing damoB in the system but barely affected the anammox population abundance (Figure 5. 8A), because of the high ammonium load compared to CH_4 load (41.7 g N m^{-3} h^{-1} and 10.4 g COD m^{-3} h^{-1}, respectively) and the relatively high yield of anammox bacteria (0.170 g COD (g N)$^{-1}$) compared to damoB (0.0835 g COD (g COD)$^{-1}$). The presence of MOB and damoA increased with increasing K_{NO2_damoB}, while the overall CH_4 removal efficiency was not affected (Figure 5. 8B). In this study, anammox bacteria are assumed to have a higher nitrite affinity than damoB (K_{NO2_damoB} = 0.6 g N m^{-3}; K_{NO2_AN} = 0.005 g N m^{-3}). Chen et al. (2015) assumed the opposite, favouring damoB when competing with anammox bacteria for nitrite (K_{NO2_damoB} = 0.01 g N m^{-3}; K_{NO2_AN} = 0.05 g N m^{-3}). The presence of damoB is influenced by K_{NO2_damoB}, as shown in this study (Figure 5. 8B) and higher values of K_{NO2_damoB} in the study of Chen et al. (2015) (more advantageous situation for anammox bacteria than for damoB) could have changed the CH_4 removal in their system.

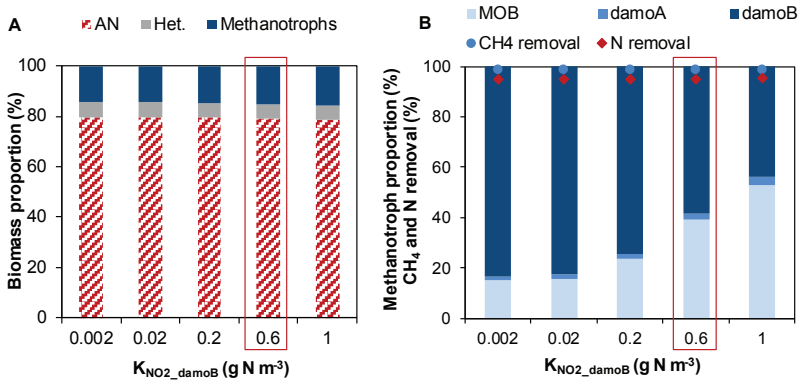

Figure 5. 8 Effect of nitrite half saturation constant for damoB on the relative abundance of populations competing with damoB for substrate: A) competition with anammox bacteria and anaerobic heterotrophs (HNO_2) for nitrate and B) competition with damoA and MOB for CH_4. Influent NH_4^+ = 400 g N m^{-3}; DO = 0.2 g O_2 m^{-3}.

5.3.5 Effect of reactor operation and design on CH_4 stripping

The effect of aeration intensity (k_La) on the fate of CH_4 is summarized in Figure 5. 9. High nitrogen removal was achieved at the expense of high CH_4 stripping when applying surface aeration (maximum nitrogen removal achieved = 98% at k_La_{O2} = 500 d^{-1} with 56.5% of CH_4 stripped, Figure 5. 9).

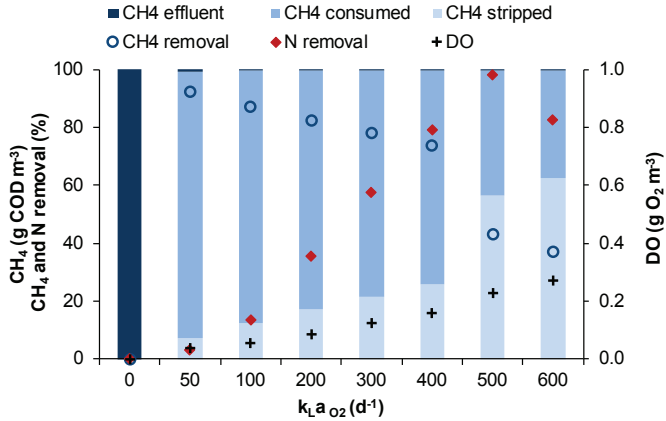

Figure 5. 9 Effect of aeration intensity (applying surface aeration) on the fraction of the total CH_4 fed (100 g COD m^{-3}) which is stripped, consumed or ends up in the effluent. The corresponding nitrogen removal efficiencies and DO concentrations are also displayed. Results obtained for an influent ammonium concentration = 700 g NH_4^+-N m^{-3}.

CH_4 stripping could be reduced through partial recirculation of the off-gas, as already applied in certain full-scale aerobic anammox-based granular sludge reactors as a means of aeration control (see Chapter 4). Recirculation of part of the off-gas would increase the CH_4 partial pressure and thus lead to less CH_4 stripping. However, CH_4 in the non-recirculated part of the off-gas would still end up in the atmosphere. A second option to minimize CH_4 stripping would be to use subsurface aeration rather than surface aeration (Daelman et al. 2014) and to locate the (CH_4 containing) influent supply line inside the reactor. Installing the aeration supply deeper in the tank results in an increased gas solubility (CH_4 and O_2), implying less CH_4 stripping, but is counteracted by a stronger saturation (in CH_4) or depletion (in O_2) of the rising gas bubbles (higher gas retention time). Figure 5. 10 summarizes the effect of the depth of aeration supply on the CH_4 stripped, removed and not converted (in the effluent), for the cases with the highest nitrogen removal efficiency (details on the underlying simulations are given in the Appendix (Figure A5. 5). CH_4 stripped was reduced from 56% to 23% (i.e., by 60%) while keeping the same nitrogen removal efficiency (98%) when increasing the aeration supply depth from 0 (i.e. case of surface aeration) to 8 m (Figure 5. 10). A further increase (to 12 m) only slightly reduced the CH_4 stripping

and increased the CH_4 conversion (from 77% to 80% CH_4 converted for an aeration depth of 8 and 12 m, respectively), which may not be warranted by the increased DO concentration needed to achieve this result, also considering that higher energy costs will be required to supply oxygen at this depth.

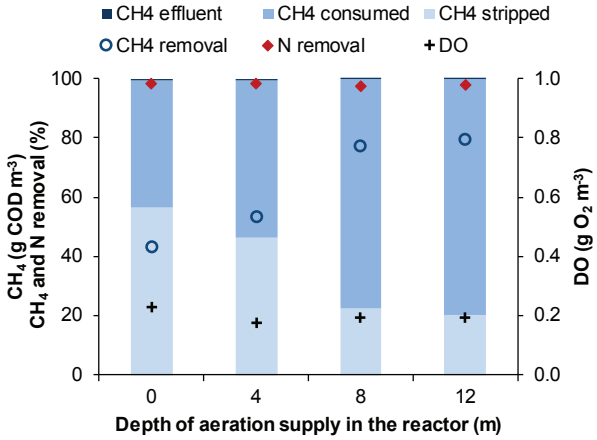

Figure 5. 10 Influence of the depth of aeration supply (liquid column height) on dissolved CH_4 in the effluent, CH_4 consumed and CH_4 stripped, as well as corresponding CH_4 and nitrogen removal. Results obtained for maximum nitrogen removal scenarios for each depth (k_La_{O2} = 500, 450, 400, and 375 for aeration depth = 0, 4,8 and 12 m, respectively), influent NH_4^+ = 700 g N m^{-3}, Q_{inf} = 1000 m^3 d^{-1}.

5.4 Conclusions

The possible integration of CH_4 removal in an aerobic anammox-based granular sludge reactor was assessed through a simulation study.

- For this purpose, a model was set up, including not only damo bacteria and archaea, but also aerobic methane oxidizing bacteria as a potentially important microbial group for CH_4 removal, besides the bacterial populations playing a role in nitrogen removal.

- Simultaneous nitrogen and CH_4 removal was demonstrated feasible for low oxygen concentrations (< 0.5 g O_2 m^{-3}), achieving up to 99% removal efficiencies for both substrates in an ideal case without CH_4 stripping.

- While nitrogen removal efficiency was found very sensitive to the dissolved oxygen concentration, maximum CH_4 removal was obtained in

a broader oxygen concentration interval. This dissolved oxygen concentration interval always included the dissolved oxygen for maximum nitrogen removal, thus, maximum CH_4 removal was achieved under the same dissolved oxygen conditions as for maximum nitrogen removal.

- The presence of damo archaea improved the nitrogen removal efficiency by converting the nitrate formed by anammox bacteria.
- High CH_4 removal efficiencies were maintained at different influent ammonium and dissolved oxygen concentrations and when varying kinetic parameters. While a shift could be noted in the methane oxidizing population, aerobic methane oxidizing bacteria were the main responsible microorganisms for CH_4 removal in all cases.
- CH_4 stripping during aeration could be limited by increasing the aeration supply depth within the reactor (23% CH_4 stripped at 8 m depth), while keeping the nitrogen removal efficiency high.

Acknowledgements

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Appendix

A5.1 Model for simultaneous nitrogen and methane removal

The model kinetics and stoichiometry are displayed in Table A5. 1 and Table A5. 2, respectively; Table A5. 3 summarizes the corresponding parameter values.

Table A5. 1 Kinetic expressions modelled.

Process	Kinetic expressions
AOB	
1. Growth of AOB	$\mu_{max}^{AOB} \cdot \frac{S_{NH}}{K_{NH}^{AOB} + S_{NH}} \cdot \frac{S_{O2}}{K_{O2}^{AOB} + S_{O2}} \cdot \frac{K_{i-NO2}^{AOB}}{K_{i-NO2}^{AOB} + S_{NO2}} \cdot X_{AOB}$
2. Decay of AOB	$b^{AOB} \cdot X_{AOB}$
NOB	
3. Growth of NOB	$\mu_{max}^{NOB} \cdot \frac{S_{NH}}{K_{NH}^{NOB} + S_{NH}} \cdot \frac{S_{O2}}{K_{O2}^{NOB} + S_{O2}} \cdot \frac{S_{NO2}}{K_{NO2}^{NOB} + S_{NO2}} \cdot X_{NOB}$
4. Decay of NOB	$b^{NOB} \cdot X_{NOB}$
Anammox bacteria	
5. Growth of AN	$\mu_{max}^{AN} \cdot \frac{S_{NH}}{K_{NH}^{AN} + S_{NH}} \cdot \frac{S_{NO2}}{K_{NO2}^{AN} + S_{NO2}} \cdot \frac{K_{i-NO2}^{AN}}{K_{i-NO2}^{AN} + S_{NO2}} \cdot \frac{K_{i-O2}^{AN}}{K_{i-O2}^{AN} + S_{O2}} \cdot X_{AN}$
6. Decay of AN	$b^{AN} \cdot X_{AN}$
Damo bacteria	
7. Growth of damoB	$\mu_{max}^{damoB} \cdot \frac{S_{CH4}}{K_{CH4}^{damoB} + S_{CH4}} \cdot \frac{S_{NO2}}{K_{NO2}^{damoB} + S_{NO2}} \cdot \frac{K_{i-NO2}^{damoB}}{K_{i-NO2}^{damoB} + S_{NO2}} \cdot \frac{K_{i-O2}^{damoB}}{K_{i-O2}^{damoB} + S_{O2}} \cdot X_{damoB}$
8. Decay of damoB	$b^{damoB} \cdot X_{damoB}$
Damo archaea	
9. Growth of damoA	$\mu_{max}^{damoA} \cdot \frac{S_{NH}}{K_{NH}^{damoA} + S_{NH}} \cdot \frac{S_{CH4}}{K_{CH4}^{damoA} + S_{CH4}} \cdot \frac{S_{NO2}}{K_{NO2}^{damoA} + S_{NO2}} \cdot \frac{K_{i-NO2}^{damoA}}{K_{i-NO2}^{damoA} + S_{NO2}} \cdot \frac{K_{i-O2}^{damoA}}{K_{i-O2}^{damoA} + S_{O2}} \cdot X_{damoA}$
10. Decay of damoA	$b^{damoB} \cdot X_{damoB}$
MOB	
11. Growth of MOB	$\mu_{max}^{MOB} \cdot \frac{S_{NH}}{K_{NH}^{MOB} + S_{NH}} \cdot \frac{S_{O2}}{K_{O2}^{MOB} + S_{O2}} \cdot \frac{S_{CH4}}{K_{CH4}^{MOB} + S_{CH4}} \cdot X_{MOB}$
12. Decay of MOB	$b^{MOB} \cdot X_{MOB}$
Heterotrophs	
13. Aerobic growth of heterotrophs	$\mu_{max}^{H} \cdot \frac{S_{NH}}{K_{NH}^{H} + S_{NH}} \cdot \frac{S_{O2}}{K_{O2}^{H} + S_{O2}} \cdot \frac{S_S}{K_S^{H} + S_S} \cdot X_{H,A}$
14. Anoxic (nitrite) growth of heterotrophs	$\mu_{max}^{H} \cdot \eta \cdot \frac{S_{NH}}{K_{NH}^{H} + S_{NH}} \cdot \frac{S_{NO2}}{K_{NO2}^{H} + S_{NO2}} \cdot \frac{S_{NO2}}{S_{NO2} + S_{NO3}} \cdot \frac{S_S}{K_S^{H} + S_S} \cdot \frac{K_{i-O2}^{H}}{K_{i-O2}^{H} + S_{O2}} \cdot X_{H,NO2}$
15. Anoxic (nitrate) growth of heterotrophs	$\mu_{max}^{H} \cdot \eta \cdot \frac{S_{NH}}{K_{NH}^{H} + S_{NH}} \frac{S_{NO3}}{K_{NO3}^{H} + S_{NO3}} \cdot \frac{S_{NO3}}{S_{NO2} + S_{NO3}} \cdot \frac{S_S}{K_S^{H} + S_S} \cdot \frac{K_{i-O2}^{H}}{K_{i-O2}^{H} + S_{O2}} \cdot X_{H,NO3}$
16. Decay of heterotrophs	$b^{H} \cdot X_{H,A} + b^{H} \cdot X_{H,NO2} + b^{H} \cdot X_{H,NO3}$

Table A5.2 Stoichiometric matrix.

Comp. →	S_{NH}	S_{NO2}	S_{NO3}	S_{O2}	S_{N2}	S_{CH4}
Process ↓	g N m^{-3}	g N m^{-3}	g N m^{-3}	g O_2 m^{-3}	g N m^{-3}	g COD m^{-3}
AOB						
1	$-\frac{1}{Y_{AOB}} - i_{NXB}$	$\frac{1}{Y_{AOB}}$		$1-\frac{3.43}{Y_{AOB}}$		
2	$i_{NXB} - f_I i_{NXI} - (1-f_I) i_{NSS}$					
NOB						
3	$-i_{NXB}$	$-\frac{1}{Y_{NOB}}$	$\frac{1}{Y_{NOB}}$	$1-\frac{1.14}{Y_{NOB}}$		
4	$i_{NXB} - f_I i_{NXI} - (1-f_I) i_{NSS}$					
Anammox bacteria						
5	$-\frac{1}{Y_{AN}} - i_{NXB}^{AN}$	$-\frac{1}{Y_{AN}} - \frac{1}{1.14}$	$\frac{1}{1.14}$		$\frac{2}{Y_{AN}}$	
6	$i_{NXB}^{AN} - f_I i_{NXI} - (1-f_I) i_{NSS}$					
Damo bacteria						
7		$-\frac{1}{Y_{\mathrm{damoB}}} + i_{NXB}$			$\frac{1}{Y_{\mathrm{damoB}} - 2i_{NXB}}$	$-\frac{1.71}{Y_{\mathrm{damoB}}} - 1$
8	$i_{NXB} - f_I i_{NXI} - (1-f_I) i_{NSS}$					
Damo archaea						
9	$-\mathrm{i_{NXB}}$	$\frac{1}{1.14} \cdot \left(\frac{1}{\mathrm{Y_{damoA}}} - 1\right)$	$\frac{-1}{1.14} \cdot \left(\frac{1}{\mathrm{Y_{damoA}}} - 1\right)$			$-\frac{1}{\mathrm{Y_{damoA}}}$
10	$\mathrm{i_{NXB} - f_I i_{NXI} - (1-f_I) i_{NSS}}$					
MOB						
11	$-i_{NXB}$			$1-\frac{1}{Y_{MOB}}$		$-\frac{1}{Y_{MOB}}$
12	$i_{NXB} - f_I i_{NXI} - (1-f_I) i_{NSS}$					
Heterotrophs						
13	$-i_{NXB} + \frac{1}{Y_{H,A}} i_{NSS}$			$1-\frac{1}{Y_{H,A}}$		
14	$-i_{NXB} + \frac{1}{Y_{H,NO2}} i_{NSS}$	$-\frac{1-Y_{H,NO2}}{1.71 Y_{H,NO2}}$			$\frac{1-Y_{H,NO2}}{1.71 Y_{H,NO2}}$	
15	$-i_{NXB} + \frac{1}{Y_{H,NO3}} i_{NSS}$	$\frac{1-Y_{H,NO3}}{1.14 Y_{H,NO3}}$	$-\frac{1-Y_{H,NO3}}{1.14 Y_{H,NO3}}$			
16	$3(i_{NXB} - f_I i_{NXI} - (1-f_I) i_{NSS})$					

S_S	X_{AOB}	X_{NOB}	X_{AN}	X_{damoB}	X_{damoA}	X_{MOB}	X_H			X_I
g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	g COD m^{-3}	$X_{H,A}$	$X_{H,NO2}$	$X_{H,NO3}$	g COD m^{-3}
							g COD m^{-3}			
	1									
$1 - f_I$	-1									f_I
		1								
$1 - f_I$		-1								f_I
			1							
$1 - f_I$			-1							f_I
				1						
$1 - f_I$				-1						f_I
					1					
$1 - f_I$					-1					f_I
						1				
$1 - f_I$						-1				f_I
$-\frac{1}{Y_{H,A}}$							1			
$-\frac{1}{Y_{H,NO2}}$								1		
$-\frac{1}{Y_{H,NO3}}$									1	
$3(1 - f_I)$							-1	-1	-1	f_I

Table A5. 3 Stoichiometric and kinetic (at 30°C) parameter values applied in the model.

Parameter	Value	Unit		Reference
AOB				
Y_{AOB}	0.20	g COD.g^{-1} N	Yield of AOB on ammonia	(Wiesmann 1994) (1)
μ_{max}^{AOB}	1.36	d^{-1}	Maximum growth rate of AOB	(Hellinga et al. 1999) (3)
K_{NH}^{AOB}	1.1	g N.m^{-3}	Ammonium half saturation constant for AOB	(Wiesmann 1994) (5)
K_{O2}^{AOB}	0.3	g O_2.m^{-3}	Oxygen half saturation constant for AOB	(Wiesmann 1994)
K_{I-NO2}^{AOB}	846.5	g N.m^{-3}	Nitrite inhibition constant for AOB	(Pambrun et al. 2006) (5)
b^{AOB}	0.068	d^{-1}	Specific biomass decay rate of AOB	Assumed, set to $0.05\mu_{max}^{AOB}$ (Volcke et al. 2010)
NOB				
Y_{NOB}	0.057	g COD.g^{-1} N	Yield of NOB on nitrite	(Wiesmann 1994) (1)
μ_{max}^{NOB}	0.79	d^{-1}	Maximum growth rate of NOB	(Hellinga et al. 1999) (3)
K_{NH}^{NOB}	0.058	g N.m^{-3}	Ammonium half saturation constant for NOB	(Grady 2011)
K_{O2}^{NOB}	1.1	g O_2.m^{-3}	Oxygen half saturation constant for NOB	(Wiesmann 1994)
K_{NO2}^{NOB}	0.51	g N.m^{-3}	Nitrite half saturation constant for NOB	(Wiesmann 1994) (5)
b^{NOB}	0.04	d^{-1}	Specific biomass decay rate of NOB	Assumed, set to $0.05\mu_{max}^{NOB}$ (Volcke et al. 2010)
Anammox bacteria				
Y_{AN}	0.17	g COD.g^{-1} N	Yield of AN on ammonium	(Strous et al. 1998) (2)
μ_{max}^{AN}	0.052	d^{-1}	Maximum growth rate of AN	(Strous et al. 1998) (3)
K_{NH}^{AN}	0.037	g N.m^{-3}	Ammonium half saturation constant for AN	Assumed that ratio K_{NH}^{AOB}:K_{NH}^{AN} is 30 as in (Hao et al. 2002)
K_{NO2}^{AN}	0.005	g N.m^{-3}	Nitrite half saturation constant for AN	Assumed that ratio K_{NO2}^{NOB}:K_{NO2}^{AN} is 110 as in (Hao et al. 2002)
K_{I-NO2}^{AN}	400	g N.m^{-3}	Nitrite inhibition constant for AN	(Lotti et al. 2012)
K_{I-O2}^{AN}	0.01	g O_2.m^{-3}	Oxygen inhibition constant for AN	(Hao et al. 2002)
b^{AN}	0.0026	d^{-1}	Specific biomass decay rate of AN	Assumed, set to $0.05\mu_{max}^{AN}$ (Volcke et al. 2010)
Damo bacteria				
Y_{damoB}	0.156	g COD.g^{-1} N	Yield of damoB on nitrite	(Winkler et al. 2015)
μ_{max}^{damoB}	0.0495	d^{-1}	Maximum growth rate of damoB	Calculated using the reference value of (Ettwig et al. 2010) (4)
K_{CH4}^{damoB}	0.19	g COD.m^{-3}	Methane half saturation constant for damoB	Average value from the one reported by (Raghoebarsing et al. 2006) and (Ettwig et al. 2008)
K_{NO2}^{damoB}	0.6	g N.m^{-3}	Nitrite half saturation constant for damoB	(He et al. 2013)
K_{I-NO2}^{damoB}	40	g N.m^{-3}	Nitrite inhibition constant for damoB	(He et al. 2013), (Rasigraf et al. 2012)
K_{I-O2}^{damoB}	0.01	g O_2.m^{-3}	Oxygen inhibition constant for damoB	Assumed to be equal to K_{I-O2}^{AN}
b^{damoB}	0.0025	d^{-1}	Specific biomass decay rate of damoB	Assumed, set to $0.05\mu_{max}^{damoB}$
Damo archaea				
Y_{damoA}	0.071	g COD.g^{-1} COD	Yield of damoA on CH_4	(Rasigraf et al. 2012)
μ_{max}^{damoA}	0.03624	d^{-1}	Maximum growth rate of damoA	(Chen et al. 2014)
K_{NH}^{damoA}	0.02	g N.m^{-3}	Ammonium half saturation constant for damoA	Assumed in this study
K_{CH4}^{damoA}	0.19	g COD.m^{-3}	Methane half saturation constant for damoA	Same value as for damoB
K_{NO3}^{damoA}	0.11	g N.m^{-3}	Nitrate half saturation constant for damoA	(Chen et al. 2014)
K_{I-NO2}^{damoA}	40	g N.m^{-3}	Nitrite inhibition constant for damoA	Same value as for damoB
K_{I-O2}^{damoA}	0.01	g O_2.m^{-3}	Oxygen inhibition constant for damoA	Same value as for damoB
b^{damoA}	0.001812	d^{-1}	Specific biomass decay rate of damoA	Assumed, set to $0.05\mu_{max}^{damoA}$
MOB				
Y_{MOB}	0.2	g COD.g^{-1} COD	Yield of MOB on CH_4	(Arcangeli & Arvin 1999)
μ_{max}^{MOB}	3.5	d^{-1}	Maximum growth rate of MOB	(Arcangeli & Arvin 1999)
K_{NH}^{MOB}	2	g N.m^{-3}	Ammonim half saturation constant for MOB	(Arcangeli & Arvin 1997)
K_{O2}^{MOB}	0.2	g O_2.m^{-3}	Oxygen half saturation constant for MOB	(Arcangeli & Arvin 1997)
K_{CH4}^{MOB}	0.26	g COD.m^{-3}	Methane half saturation constant for MOB	(Arcangeli & Arvin 1999)
b^{MOB}	0.175	d^{-1}	Specific biomass decay rate of MOB	Assumed, set to $0.05\mu_{max}^{MOB}$

Parameter	Value	Unit		Reference
Heterotrophs				
$Y_{H,A}$	0.67	g COD.g^{-1} COD	Yield of aerobic heterotrophic bacteria	(Henze et al. 2000) (ASM1)
$Y_{H,NO2}$	0.53	g COD.g^{-1} COD	Yield of anoxic heterotrophic bacteria on nitrite	(Muller et al. 2003)
$Y_{H,NO3}$	0.53	g COD.g^{-1} COD	Yield of anoxic heterotrophic bacteria on nitrate	(Muller et al. 2003)
μ_{max}^{H}	12	d^{-1}	Max. growth rate of heterotrophs	(Henze et al. 2000) (ASM1)
K_{NH}^{H}	0.02	g N.m^{-3}	Ammonium half saturation constant for heterotrophs	Assumed in this study
K_{O2}^{H}	0.2	g O_2.m^{-3}	Oxygen half saturation constant for heterotrophs	(Henze et al. 2000) (ASM1)
K_{S}^{H}	4	g COD.m^{-3}	Organic substrate half saturation constant for heterotrophs	(Alpkvist et al. 2006)
K_{NO2}^{H}	0.3	g N.m^{-3}	Nitrite half saturation constant for heterotrophs	(Alpkvist et al. 2006)
K_{NO3}^{H}	0.3	g N.m^{-3}	Nitrate half saturation constant for heterotrophs	(Alpkvist et al. 2006)
K_{i-O2}^{H}	0.35	g O_2.m^{-3}	Oxygen inhibition constant for heterotrophs	(Alpkvist et al. 2006)
b^{H}	0.6	d^{-1}	Specific biomass decay rate of heterotrophs	Assumed, set to $0.05\mu_{max}^{H}$
i_{NXB}	0.083	g N.g^{-1} COD	Nitrogen content in active biomass	For composition $CH_{1.8}O_{0.5}N_{0.2}$ (Henze et al. 2008)
i_{NXB}^{AN}	0.058	g N.g^{-1} COD	Nitrogen content in active biomass of AN	For composition $CH_2O_{0.5}N_{0.15}$ (Strous et al. 1998)
i_{NXI}	0.06	g N.g^{-1} COD	Nitrogen content in X_I	(Henze et al. 2000) (ASM1)
i_{NSS}	0.03	g N.g^{-1} COD	Nitrogen content in organic substrate	(Henze et al. 2000) (ASM3)
f_I	0.08	g COD.g^{-1} COD	Inert content in biomass	(Henze et al. 2000) (ASM1)
η	0.8	-	Correction factor for μ^{H} under anoxic conditions	(Henze et al. 2000) (ASM1)

(1) After unit conversion, for a typical biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$, corresponding to 1.3659 g COD g^{-1} (Henze et al., 2008).

$Y_{AOB} = 0.147$ g biomass g^{-1} N $= 0.201$ g COD g^{-1} N

$Y_{NOB} = 0.042$ g biomass g^{-1} N $= 0.057$ g COD g^{-1} N

(2) After unit conversion, for an anammox biomass composition of $CH_2O_{0.5}N_{0.15}$ (Strous et al., 1998) corresponding to 1.51 g COD g^{-1} biomass.

(3) Conversions of maximum growth rate of AOB, NOB, anammox bacteria, and MOB at different temperatures to 30 ^{o}C through the following relationship (Jetten et al. 1997).

$$\mu_{max}^{AOB}(T) = \mu_{max}^{AOB}(T_{ref}) exp\left(\frac{E_a^{AOB}(T - T_{ref})}{RTT_{ref}}\right)$$

with: $E_a^{AOB} = 68\ kJ\ mol^{-1}$; $E_a^{NOB} = 44\ kJ\ mol^{-1}$; at $T_{ref} = 35\ ^{o}$C (Hellinga et al., 1999). $E_a^{AN} = 70\ kJ\ mol^{-1}$ at $T_{ref} = 32.5^{o}$C (Strous et al. 1999). $E_a^{MOB} = 59.7\ kJ\ mol^{-1}$ (Cai & Yan 1999) and $E_a^{MOB} = 56.5\ kJ\ mol^{-1}$ (Boeckx & VanCleemput 1996). The average value $E_a^{MOB} = 58.1\ kJ\ mol^{-1}$ was used at $T_{ref} = 20\ ^{o}C$.

(4) According to Ettwig et al. (2010), mesophilic damoB at 25–30 ^{o}C and pH 7–8 have a slow growth (doubling time 1–2 weeks). A doubling time of 2 weeks, i.e. the most unfavourable situation, corresponds to $\mu_{max}^{damoB} = \frac{ln2}{t_d} = 0.0495\ d^{-1}$

(5) Conversion from the values from the non-ionized components to the ionized components according to the chemical equilibriums: $NH_4^+ \leftrightarrow NH_3 + H^+$ and $HNO_2 \leftrightarrow NO_2^- + H^+$.

A5.2 Simulation results concerning the influence of influent ammonium and bulk oxygen concentrations

A series of simulations were carried out concerning the influence of the bulk oxygen concentration at different influent ammonium concentrations. The resulting reactor performance in terms of effluent substrate concentrations, and removal efficiencies are displayed in Figures A5. 1 and A5. 2, respectively.

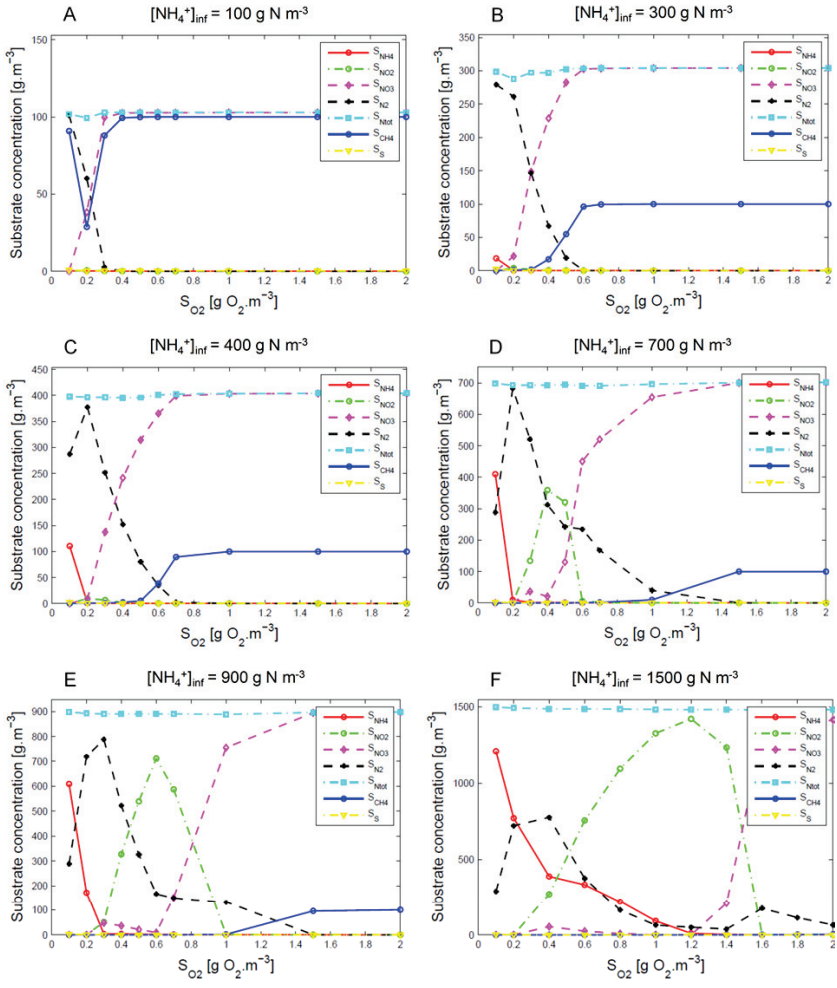

Figure A5. 1 Influence of the bulk oxygen concentration on the reactor performance in terms of effluent substrate concentrations at different influent ammonium concentrations.

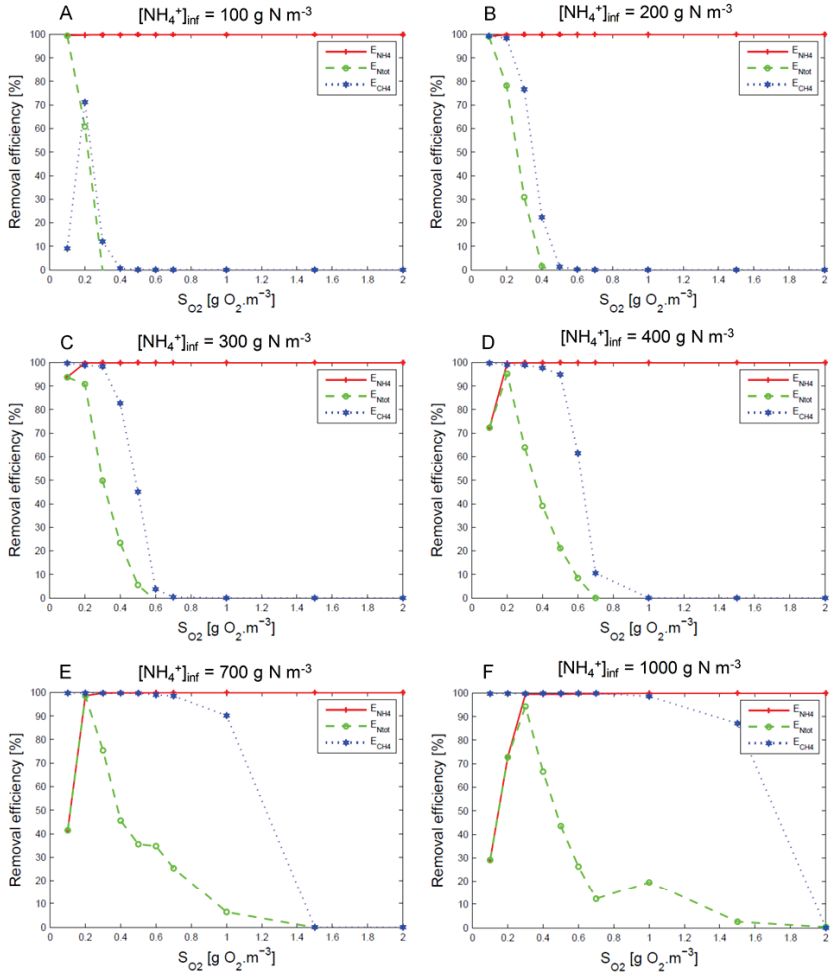

Figure A5. 2 Influence of the bulk oxygen concentration on the reactor performance in terms of ammonium (E_{NH4}), total nitrogen (E_{Ntot}) and CH_4 (E_{CH4}) removal efficiencies, at different influent ammonium concentrations.

A5.3. Contribution to nitrogen removal by microbial populations

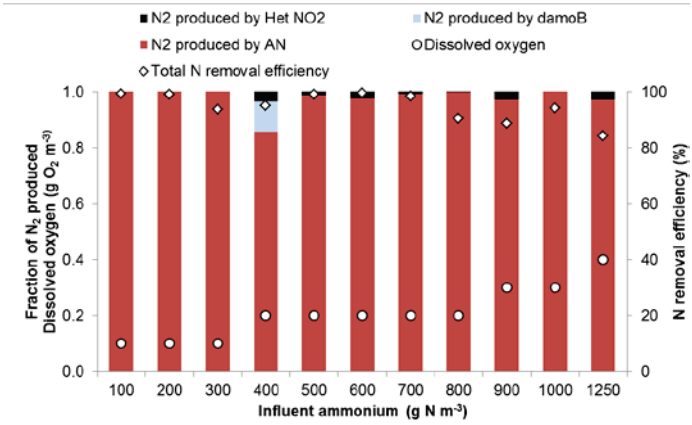

Figure A5. 3 Contribution of anammox bacteria, damoB and heterotrophs (converting NO_2^-) to the production of N_2 in terms of the influent ammonium concentration, for the scenarios with maximum N removal efficiency.

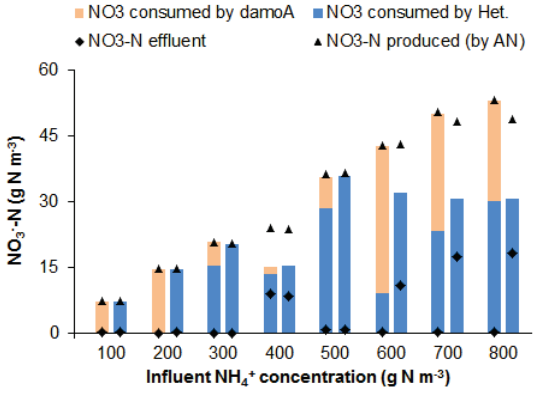

Figure A5. 4 Microbial contributions to NO_3^- consumption with damoA in the system (left bar) and when running simulations suppressing the damoA process in the system (right bar), in function of influent NH_4^+ concentration. Bulk oxygen concentration 0.1 g O_2 m^{-3} for 100-300 g NH_4^+-N m^{-3} and 0.2 g O_2 m^{-3} for 400-800 g NH_4^+-N m^{-3}.

A5.4. Nitrogen calculation

Table A5. 4 Nitrogen balance to assess the nitrite availability for damoB for an influent ammonium concentration of 400 g NH_4^+-N m^{-3}.

	NH_4^+ consumed (g N)	NO_2^- produced or consumed (negative values) (g N)	CH_4 consumed (g COD)
AOB	238.8*	234.9*	
Anammox	400-238.8 = 161.2	-182.7	
Remaining	0	52.2	
DamoB	-	- 41.9	79.2*
Remaining	-	10.3 (9.6*)	

* Data from simulation

Considering that all available ammonium after AOB consumption is taken by anammox (worst-case scenario that does not consider the ammonium uptake by other populations to grow, corresponding with highest ammonium uptake and thus highest corresponding nitrite uptake by anammox), there would still be enough nitrite for damoB to consume all available CH_4.

A5.5. CH_4 stripping in function of aeration depth

To assess the CH_4 stripping in the aerated granular sludge system, the gas-liquid transfer rate for oxygen and liquid-gas for CH_4 (TR_i) was included in the model. The influence of the depth where the aerators where located in the mass transfer was taken into account as in Daelman et al. (2014), resulting in the following equation A5.1.

$$TR_i = k_L a_i \cdot \frac{\frac{(p_t + \frac{1}{2} \cdot \rho \cdot g \cdot H) \cdot M_i}{m_i \cdot R \cdot T^0} \cdot x_{i,in} - S_i}{1 + \frac{0.6 \cdot H}{m_i}} \qquad [A5.1]$$

Where:

g	Gravitational field constant	m s^{-2}
H	Height of the column	m
$k_L a_i$	Gas-liquid mass transfer coefficient of compound i	s^{-1}

m_i	Partition coefficient gas-liquid $\frac{c_G}{S_i^*}$	-
M_i	Molecular weight of compound i	kg mol^{-1}
p_t	Pressure at the water surface	Pa
R	Ideal gas constant	Pa m^3 mol^{-1} K^{-1}
S_i	Concentration of compound i in the mixed liquor	kg m^{-3}
T^0	Temperature at reference conditions	K
TR_i	Transfer rate of compound i	kg m^{-3} d^{-1}
$x_{i,in}$	Concentration of compound i in the ingoing aeration gas flow (mole fraction)	-
$x_{i,out}$	Concentration of compound i in the outgoing aeration gas flow (mole fraction)	-
ρ	Density of the mixed liquor	kg m^{-3}

This equation can be applied for bubble columns with a height:diameter ratio < 3.

The volumetric mass transfer coefficient k_La for oxygen (K_La_{O2}) was fixed for the simulations while the one for CH_4 (K_La_{CH4}) was determined according to Eq. A5.2, where D_i is the diffusivity at 30 °C in water for each gas.

$$k_L a_{CH_4} = k_L a_{O_2} \cdot \sqrt{\frac{D_{CH_4}}{D_{O_2}}} \qquad [A5.2]$$

The results of the CH_4 stripped considering different column heights is displayed in Figure A5. 6.

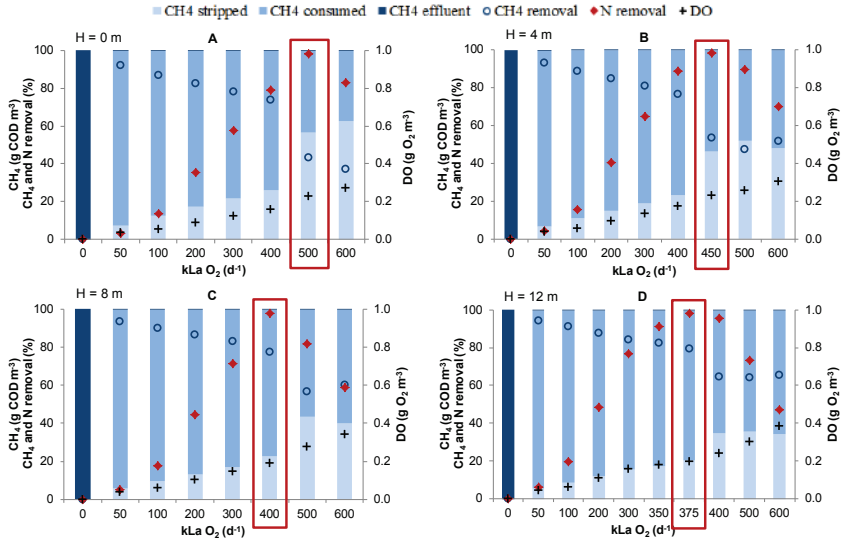

Figure A5. 5 Dissolved CH_4 in the effluent, CH_4 consumed and stripped, N and CH_4 removal efficiencies at different aeration intensities (k_La O_2) when locating the aeration at A) 0 m, B) 4 m, C) 8 m and D) 12 m column height. Influent ammonium = 700 g N m^{-3}, Q_{inf} = 1000 m^3 d^{-1}. Optimal cases in red squares are displayed in Figure 10.

A5.6. Calculation of the carbon footprint

The aeration energy associated with oxygen supply to aerobic methane oxidizers entails CO_2 emissions. A calculation was made to determine if this contribution to the carbon footprint is comparable to the carbon footprint related to the direct emission of CH_4.

Table A5. 5 Data used for the calculation. Due to the higher emission impact, petroleum coke was chosen for the calculation, since this would be one of the worst scenarios.

1000	g O_2/kWh	Specific energy consumption associated to oxygen transfer (Garrido et al. 2013)
1000	g CO_2/kWh	Considering petroleum coke as fuel (International Energy Agency)
400	g CO_2/kWh	Considering natural gas (International Energy Agency)
34	global warming potential of CH_4, CO_2 equivalents	

The calculation was based in 1 m^3 of wastewater with a dissolved concentration of 25 g CH_4 m^{-3}. The results are presented in Table A5. 6. The carbon footprint (CO_2 equivalents) of the CH_4 emitted directly would be significantly higher than the CO_2 associated to the oxidation of CH_4.

Table A5. 6 Calculation of the CO_2 equivalents from the combustion of CH_4 and the direct emission of the greenhouse gas. Calculation based in 1 m^3 of wastewater with a concentration of 25 g CH_4 m^{-3}.

CO_2 from fuel combustion and oxidation of CH_4 to CO_2

25	g	CH_4	→	100	g CO_2	due to petroleum combustion
			→	69	g CO_2	due to the CH_4 oxidation to CO_2
			Total	169	g CO_2	

CO_2 equivalents if the CH_4 is emitted directly

25	g	CH4	→	850	g CO_2

6

Conclusions and perspectives

The objective of this thesis was to contribute to the reduction of the greenhouse gases CO_2, N_2O and CH_4 during nitrogen removal from wastewater treatment through the optimization of process operation and the application of innovative anammox-based technologies. Both municipal wastewater (cold and low strength ammonium wastewater; Chapters 2 and 3) and sidestream wastewater (warm and rich-ammonium wastewater; Chapter 4 and 5) were considered for the study. The methodology used involved lab-scale experiments (Chapter 2 and 3), a full-scale monitoring campaign (Chapter 4) as well as mathematical modelling and simulation (Chapter 5). This chapter summarizes the key outcomes from this work and gives indications for future research.

CO_2 reduction

The main source of CO_2 emissions from WWTP is the combustion of fossil fuels to generate energy for the plant. The aeration energy needed for nitrogen and COD removal is one of the most demanding factors in this respect. **Anammox-based technologies** are more sustainable nitrogen removal alternatives compared to conventional nitrification-denitrification over nitrate processes since they can save up to 63% energy in aeration or could even yield energy if applied in the mainstream of the WWTP (Kartal et al. 2010). Also, the anammox reaction does not catabolize the organic matter present in the wastewater, which energy or chemical potential could be recovered and maximized, instead of oxidizing it to CO_2 by heterotrophic microorganisms. However, the implementation of the anammox conversion in the **mainstream** is being very challenging. One of the main bottlenecks is an effective supply of nitrite to the anammox reaction (i.e. the suppression of NOB) (Lotti et al. 2015; Ma et al. 2016).

In this regard, Chapter 2 proposed the application of the **organotrophic nitrate reduction capacity of anammox bacteria** (DNRA) together with the regular anammox transformation as an innovative nitrogen removal system for mainstream wastewater. In this approach the suppression of NOB would not be so crucial since anammox bacteria could reduce the nitrate formed by

NOB to nitrite, while oxidizing organic compounds (partial DNRA) and then would combine the nitrite obtained and the ammonium contained in the wastewater to yield nitrogen gas (anammox reaction). In this study *Candidatus* 'Brocadia fulgida' was found to perform partial DNRA-anammox showing a high nitrate reduction to nitrite performance, even if the culture was not previously adapted to the supply of organic carbon compounds (acetate). Thus, this could be a good species to achieve anammox enriched cultures performing partial DNRA-anammox conversion.

Different factors were studied to gain understanding on the **carbon and nitrogen utilization among anammox bacteria and heterotrophs**. The **presence of ammonium** gave advantage to DNRA by anammox over heterotrophic transformations, as also shown previously (Kartal et al. 2007b). Moreover, when ammonium was present, anammox bacteria performed partial DNRA (reduction of nitrate to nitrite only) instead of full DNRA (conversion of nitrate to ammonium), since the conversion of external ammonium during the anammox reaction is faster than forming ammonium through DNRA. From an energy perspective, **low COD/N** ratios in the influent favour partial DNRA-anammox over heterotrophic transformations, since more Gibbs free energy is harvested per mole of COD in the former case. The organic carbon limitation was also shown to benefit the anammox conversion in a system with high presence of heterotrophs. In that case, the DNRA by anammox bacteria was hindered, but the anammox conversion took place when supplying **nitrate in the system**.Mozumder et al. (2014). The limitation of **inorganic carbon** hindered the anammox conversion. Anammox bacteria do not grow on organic carbon, and their growth limitation by inorganic carbon compromises the whole partial DNRA-anammox process.

In general, all these findings on the carbon and nitrogen utilization by anammox bacteria and their competition with heterotrophs contribute to the potential implementation of the innovative partial DNRA-anammox technology, which would make the suppression of NOB no longer so required for the success of anammox process in the mainstream. Also, this system would save 50% aeration energy if compared with conventional nitrification-

denitrification systems, since up to half of the ammonium would be oxidized to nitrate, thus decreasing the electricity consumption in the plant and the CO_2 emissions.

N_2O reduction

N_2O is emitted during biological nitrogen removal from wastewater, being AOB one of the major contributors to this N_2O formation. Still, little is known about N_2O and NO emissions during nitrification of low strength ammonium wastewater. During **mainstream treatment**, nitrite accumulation may take place, for instance while trying to suppress NOB for a successful implementation of the partial nitritation-anammox system at low temperatures (Lotti et al. 2014a) or during certain operating conditions in conventional biological nitrogen removal systems. Nitrite is an important factor influencing N_2O emissions. The effect of nitrite on the production of N_2O and NO during nitrification of low-strength ammonium wastewater was evaluated in a nitrifying lab-scale reactor in Chapter 3. It was confirmed that nitrite peaks or **nitrite accumulation** during short periods of time increase the N_2O and NO emissions in municipal wastewater. Also, the ammonium oxidizers were the responsible organisms for the N_2O and NO production. Under sudden high nitrite concentrations, **NO emissions** were more significant than the N_2O emissions. The **ammonium oxidation** activity was slowed down (AOB inhibited), while the nitrite oxidation was not affected (NOB no inhibited) at high nitrite concentrations. This outcome emphasises the different response to nitrite of cultures not adapted to the accumulation of nitrite (present study) and the response of cultures adapted (nitritation reactors), in which the N_2O emissions are not affected by nitrite increase (Pijuan et al. 2014) or the ammonium oxidation is not inhibited (Law et al. 2013b).

In general, N_2O emissions are not expected from the anammox transformation, since this gas is not an intermediate in the metabolism of anammox bacteria (Kartal et al. 2011). In this sense, the implementation of anammox systems contributes to the minimization of N_2O emissions from

wastewater treatment plants. However, typically a preceding partial nitritation reaction is applied to provide nitrite for the anammox conversion. Little research has been devoted to the study concerning N_2O emissions in **one-stage full-scale partial nitritation-anammox systems** and the influence of process design and operation on the emissions is not well understood. In this regard, the **effect of aeration** on the N_2O emissions from a one-stage full-scale granular sludge reactor performing partial nitritation-anammox was studied in Chapter 4. The dynamics of the emissions were captured through online N_2O monitoring. It was shown that high aeration prompts the N_2O emissions, not only due to gas stripping enhancement, but also because of a higher N_2O formation rate. This finding contrasts with the results found in two-unit reactor configuration, in which the N_2O formation occurred mainly during anoxic periods (Mampaey et al. 2016). In the present study, anoxic periods were a sink of N_2O emissions by heterotrophic reduction of N_2O to nitrogen gas. However, the transient from anoxic (or low aerated periods) to aerobic ones promoted the formation of N_2O. These transient moments were characterized by the highest N_2O emission and formation.

The **pathways** for the N_2O formation in both studies evaluating the effect of nitrite during the nitrification of low strength ammonium wastewater (Chapter 3) and the effect of aeration in a full-scale partial nitritation-anammox reactor treating high strength ammonium wastewater (Chapter 4) were determined. For the former research, the pathway producing the N_2O and NO when nitrite was added to the reactor was the **nitrifier denitrification**, since nitrite was the precursor on the formation of both gases. Given that significant NO emissions were reached when providing high nitrite concentrations, NO cannot be neglected as intermediate during nitrifier denitrification. Although the hydroxylamine oxidation route could play an important role during the operation of the reactor when nitrite was not accumulated, the N_2O and NO emissions are not expected to be elevated in this situation due to the low ammonium oxidation (low ammonium concentration), as shown in the study. However, in the full-scale partial nitritation-anammox reactor, the **hydroxylamine oxidation pathway** was

the most likely and important responsible mechanism for the N_2O formation. The highest N_2O formation rates were found from the recovery from anoxic periods, which had an accumulation of ammonium with subsequent improvement of the ammonium consumption rate when the aeration was activated. High ammonium consumption rates together with aerobic conditions are known to promote the hydroxylamine oxidation pathway (Wunderlin et al. 2012). The possible contribution of nitrifier denitrification was not considered important because nitrite did not accumulate in this system. The heterotrophic contribution to the N_2O emissions cannot be discarded, but the relative importance of both hydroxylamine oxidation and incomplete denitrification was not determined.

The results obtained contribute to the understanding of N_2O formation and to the **minimization of the emissions** by setting and adequate reactor operation. Clearly, nitrite accumulation needs to be prevented, since the contribution to N_2O and NO emissions can be significant in cultures not adapted to nitrite as shown in a nitrifying reactor in Chapter 3. Moreover, in single-stage anammox based reactors (Chapter 4), avoiding the accumulation of nitrite also prevents N_2O emissions, besides anammox inhibition. In the absence of nitrite, the heterotrophic activity turns out positive by removing N_2O that was formed. However, in case of nitrite accumulation (partial nitritation reactor in two reactor configuration (Mampaey et al. 2016)), heterotrophs contribute to N_2O formation during anoxic periods due to the incomplete denitrification of the nitrite present. In this type of two-stage configuration, shortening the anoxic interval could minimize N_2O emissions. For single-stage reactors, the aeration intensity is an important variable influencing the N_2O emissions as well, but in an opposite way. Short aerobic periods are recommended to minimize N_2O emissions, as well as smoother shifts in the aeration. In general, the operational conditions influence the N_2O formation, but the reactor configuration (one or two stages) needs to be taken into account when establishing appropriate operational strategies.

CH_4 reduction

Reject water from anaerobic digestion contains dissolved CH_4, which can be released to the atmosphere during downstream processing, contributing significantly to the carbon footprint of the WWTP (CH_4 = 34 CO_2eq, IPCC (2013)). In Chapter 5 the **integration of CH_4 removal in aerobic anammox-based granular sludge reactors was evaluated for the first time**. A mathematical model was set up including denitrifying anaerobic methane oxidizing (damo) bacteria and archaea, aerobic methanotrophs and anammox bacteria, besides AOB, NOB and heterotrophs, in a one-stage aerobic granular sludge reactor. Simulations at different ammonium and dissolved oxygen concentrations were performed to assess the substrate competition among the communities and to gain understanding on this innovative process integration.

The simulation results showed that **simultaneous CH_4 and nitrogen removal** in aerobic granular sludge anammox-based reactors is feasible under oxygen limiting concentrations. It was found that maximum CH_4 removal can be achieved under the same conditions (dissolved oxygen concentration) that lead to maximum nitrogen removal. Specifically, **damo archaea improved the overall nitrogen removal** by converting the nitrate formed during the anammox reaction to nitrite, which can be taken up by anammox bacteria. In general, the main methanotrophs contributing to CH_4 removal were the **aerobic methane oxidizing bacteria**.

Obtaining the maximum CH_4 removal in the system did not require the presence of all methanotrophs (damo bacteria, damo archaea and aerobic methane oxidizing bacteria). Actually, the relative coexistence of the methanotrophs changed depending on the system conditions (ammonium and oxygen concentration), thus keeping a high CH_4 removal when varying the settings. Due to this **shift on the methanotrophic populations**, the variation of kinetic parameters did not affect the system performance in terms of CH_4 removal.

A priori, the highest challenge for the implementation of this system would be the potential **CH_4 stripping** due to the aeration conditions. However, it was found that the CH_4 stripping could be limited by deeper location of the aeration supply in the reactor. When using subsurface aeration, the gas solubility increases, thus reducing the CH_4 stripping.

The outcome from this simulation study gives interesting insights on the conditions that may lead to the **implementation** of this combined system in practice (methane oxidation and partial nitritation-anammox in one-stage aerobic system).

Integrating CO_2, N_2O and CH_4 reduction strategies

This thesis contributes to the sustainability of wastewater treatment plants by minimizing the emission of the greenhouse gases CO_2, N_2O and CH_4 during nitrogen removal from wastewater.

Regarding the main line, the application of the organotrophic nitrate reduction by anammox bacteria could promote the implementation of the anammox process, since it makes NOB suppression less crucial. The implementation of this combined process, where half of the ammonium is oxidized to nitrate, which is subsequently converted by anammox bacteria together with the remaining ammonium and some organic compounds to nitrogen gas, would significantly decrease the CO_2 emissions associated with the plant's energy demand by around 50% if compared to conventional nitrification-denitrification systems. Municipal wastewater contains sufficient organic compounds to carry out the reduction of nitrate to nitrite by anammox bacteria. The accumulation of nitrite in such anammox-based systems would limited, both for one or two reactor configurations, since nitrate formation is aimed at in the first stage. Therefore, the N_2O formation would not be promoted, as it would be in systems where nitrite accumulates (Chapter 3). When considering the implementation of partial nitritation-anammox in mainstream (not yet demonstrated at full-scale), an adequate aeration control in the system would minimize the N_2O formation according to Chapter 4. For single reactor configurations, smooth aeration transitions

are recommended and since the ammonium concentration is relatively low (mainstream), the introduction of short anoxic periods may favour the removal of N_2O that is formed without high ammonium accumulation. The anammox-based systems for mainstream wastewater treatment, either including the nitrate reduction to nitrite by anammox bacteria (Chapter 2) or performing partial nitritation-anammox, would receive reject water with dissolved CH_4 if anaerobic digestion is used for energy recovery in the plant. Biological CH_4 conversion could be then integrated in the anammox-based system by (Chapter 5).

A similar approach can be done when considering nitrogen removal in the sidestream. Anammox-based reactors, i.e. partial nitritation-anammox, would contribute to CO_2 minimization by reducing aeration requirements if compared to conventional nitrification-denitrification systems. N_2O could be reduced by optimizing the reactor operation: reducing the anoxic periods in the nitritation reactor when considering two-stages, avoiding anoxic periods and smothering the transition between the aeration intervals in one-stage reactors. Besides, the carbon footprint in these reactors could be reduced by integrating CH_4 removal as shown in Chapter 5.

Future research and perspectives

Normally, partial nitritation is aimed for the anammox implementation, which can give up to 63% aeration energy savings compared to full nitrification. However, systems including partial nitritation can lead to relatively high N_2O emissions, for instance due to nitrite accumulation (Chapter 3) or to sudden changes in the aeration (Chapter 4). Current knowledge on the influence of operational parameters and reactor configuration on the N_2O emissions in full-scale partial nitritation-anammox reactors is limited. In this work the effect of aeration on the N_2O emissions in one-stage partial nitritation-anammox granular sludge reactor was investigated. The results found regarding the conditions promoting N_2O formation contrast with those obtained in other studies with two-stage reactor configuration (Mampaey et al. 2016). In the present research (one-unit reactor configuration), higher N_2O formation was found during high aeration periods and consumption of N_2O

was detected during anoxic intervals, while in Mampaey et al. (2016) the higher contribution to the N_2O formation occurred during anoxic periods. The reason behind is likely related to the N_2O formation mechanism, hydroxylamine oxidation in the first case and incomplete denitrification of nitrite in the second one. Therefore, more investigation is needed regarding the **influence of the reactor configuration** (one-unit and two-unit configurations) together with **operational conditions** (nitrite concentration, aeration pattern, length of anoxic periods) of full-scale partial nitritation-anammox systems on the N_2O emission and formation pathways.

The integration of the **nitrate reduction by anammox bacteria** opens a new window for the successful implementation of anammox-based technologies in the main line of the WWTP. The results obtained in Chapter 2 are a starting base for future research on this topic. More investigation on the **long term effectiveness** of the factors promoting the organotrophic activity by anammox bacteria over the heterotrophic transformations under mainstream conditions (low temperature) is required. The **COD/N ratio** seems to be a key parameter enhancing the partial DNRA-anammox by anammox bacteria. Although it was shown in Chapter 2 that the organic carbon limitation improves this combined process, specific COD/N ratios should be evaluated to determine the optimal one.

Regarding the **microbiology of the DNRA by anammox bacteria**, several aspects are not clear yet. These microorganisms oxidize volatile fatty acids via acetyl-CoA (Russ et al. 2012), which is a precursor for anabolic reactions. However, it was shown that anammox microorganisms oxidize the volatile fatty acids to CO_2. Why do they produce CO_2 if then they need to fix it through the reductive acetyl-CoA pathway (Strous et al. 2006)? The reason of this, in principle, inefficient way of using the organic acids, deserves further investigation. A hypothetical explanation could be related to the occurrence of these conversions in different locations in the cell. Also, it is not clear if anammox bacteria can harvest energy while performing the DNRA pathway since they are assumed to not grow on organic compounds.

For the implementation of a partial DNRA-anammox process one-reactor and two-reactor configurations were suggested. Further research is required on the evaluation of both schemes in terms of **practical feasibility and controllability**. The implementation of two-unit configuration could have an easy control, however the feasibility to carry out the nitrification in the first reactor could be compromised by alkalinity limitation and decrease of the pH. Recirculation from the second reactor (partial DNRA-anammox) could provide the needed alkalinity when this is not enough to perform the nitrification of half of the ammonium. Regarding the one-unit configuration, intermittent aerobic and anoxic periods would be necessary, which controllability could be more difficult than in two-unit reactor configuration.

The assessment of greenhouse gas emissions (including N_2O emissions) needs to be added as an additional criterion in the **evaluation of process sustainability** of partial DNRA-anammox systems. Partial nitrification (half of the ammonium converted to nitrate) to obtain the nitrate for the partial DNRA reaction could save up 50% aeration energy compared to full nitrification, which is less than the 63% mentioned for partial nitritation-anammox. However, in a hypothetical partial DNRA-anammox system, nitrite accumulation is not expected). An evaluation in terms of **total greenhouse gas contribution for different configurations** considering partial nitritation-anammox systems and partial nitrification together with partial DNRAanammox in different reactor configurations would also be helpful to determine the system which would give the lowest carbon footprint.

The integration of CH_4 removal in aerobic granular sludge anammox-based systems was carried out through mathematical modelling. All insights from this work can be used to **assess in practice the feasibility of the simultaneous CH_4 and nitrogen removal** in aerobic anammox-based granular sludge reactors. The implementation in lab-scale reactors of this combined process would help not only to evaluate its feasibility, but also to determine kinetic parameters and thus validate and calibrate the model. Furthermore, it was found that damo archaea increased the overall nitrogen removal by reducing nitrate to nitrite that could be removed during the

anammox conversion. Interestingly, Mozumder et al. (2014) found that heterotrophs could improve also the nitrogen removal of partial nitritation-anammox systems in a similar way when providing little amount of organic compounds. In this regard, it would be interesting to **assess the contribution by damo archaea and heterotrophs** to the total nitrogen removal when providing some organic compounds together with CH_4 in the system (i.e. the competition between heterotrophs and damo archaea for nitrate).

In this thesis, the reduction of greenhouse gas emissions was investigated to contribute to the **sustainability of wastewater treatment plants**. However, sustainability concerns more aspects besides greenhouse gas emissions, as shown for instance in the life cycle assessment of Schaubroeck et al. (2015). **Nitrogen recovery** instead of removal (this thesis) could be evaluated. It does not seem very logic to spend energy to convert the fixed nitrogen that humans excrete and end in the wastewater to nitrogen gas, and then to fix the nitrogen again. However, the market price of fertilizers and ammonia is one of the main drivers to either or not recover nitrogen from wastewater. Also, nitrogen removal can lead to energy-positive wastewater treatment plants (anammox in the main line (Kartal et al. 2010)) and is in general a more efficient solution for wastewater treatment than nutrient recovery. Regarding the energy consumption, the **electricity** that the implementation of the studied measures to reduce greenhouse gases would consume was not explicitly evaluated. The strategies suggested to reduce N_2O emissions based on the aeration control could be evaluated in terms of electricity consumption. The potential CH_4 removal in aerobic anammox-based reactors could be also examined and compared with the electricity consumption that would result when increasing the aeration supply depth within the reactor to avoid the CH_4 stripping. Overall, the reduction of greenhouse gas emissions is an important environmental factor and the mitigation strategies studied in this thesis could be included in more detailed sustainability assessments.

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Curriculum vitae

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Education

2012-… **Ph.D. candidate on applied biological sciences: Environmental Technology**
Ghent University, Ghent, Belgium
Promotor: Prof. Eveline Volcke
Topic: Greenhouse gas reduction through innovative nitrogen removal from wastewater

2010-2011 **Master Degree in Chemical and Environmental Process Engineering**
University of Santiago de Compostela, Santiago de Compostela, Spain
Master thesis: Valorisation of biodiesel production wastes to obtain bioplastics in mixed cultures

2004-2010 **Bachelor and Master Degree in Chemical Engineering**
University of Santiago de Compostela, Santiago de Compostela, Spain
Master thesis: Plant of production of biosurfactants

Professional experience

2011-2012 **Manufacturing Quality Assurance department**
Lonza Biologics Porriño, Spain
Pharmaceutical sector

Further education and training

UGent Doctoral Training Programme (Doctoral School of Bioscience Engineering)

Training weeks within the ITN Marie Curie Actions:

Decision-making and multi-criteria analysis for Urban Water Systems, University of Girona, Girona, Spain (20-24 October 2014);

Practical field work, Ghent University and Aquafin, Ghent, Belgium (21-25 October 2013)

Modelling and control of urban wastewater systems, Lund University, Lund, Sweden (22-26 October 2012)

Complementary Skills and Policy, Exeter University, Exeter, England (18-22 June 2012)

8th Advanced Biofilm Course, Helsingør, Denmark (7-12 October 2013)

Research stays

Delft University of Technology, Delft, the Netherlands (Apr-May 2015), with Prof. Mark van Loosdrecht
Research topic: Carbon utilization by anammox cultures

Catalan Institute for Water research (ICRA), Girona, Spain (Mar-Jul 2013), with Dr. Maite Pijuan
Research topic: Assessment of the effect of nitrite on the emissions of N_2O and NO from nitrifying systems

Teaching and supervising activities

Assisting the course Basics of Control Engineering, for the International Master of Science in Environmental Technology and Engineering (IMETE)

Master student supervision:

Daan Vandeplassche, Sustainable treatment of municipal wastewater through autotrophic nitrogen removal in granular sludge reactors (Aug 2015-May 2016)

Andrew Luc Lendrum, Sustainable wastewater treatment through innovative schemes involving autotrophic nitrogen removal (Feb 2014-Sep 2015)

Abhishek Anand, Anammox-based granular sludge reactors for municipal wastewater treatment (May 2014-Sep 2014)

Long Tuan Ho, Modelling and simulation of simultaneous ammonium and methane removal in aerobic granular sludge reactors (Aug 2013-Jun 2014)

Journal papers

Castro-Barros C.M., Daelman M.R.J., Mampaey K.E., van Loosdrecht M.C.M., Volcke E.I.P. (2015). Effect of aeration regime on N_2O emission from partial nitritation-anammox in a full-scale granular sludge reactor. *Water Research* **68**, 793-803

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Castro-Barros C.M., Ho L.T., Winkler M.K.-H., Volcke E.I.P. Integration of methane removal in aerobic anammox-based granular sludge reactors. *Submitted*

Castro-Barros C.M., Jia M., van Loosdrecht M.C.M, Volcke E.I.P., Winkler M.K.-H. Evaluating the potential for nitrate reduction through DNRA by anammox bacteria for municipal wastewater treatment. *Submitted*

Conference contributions

Oral contributions presented by Celia Castro Barros

Castro-Barros C.M., Ho L.T., Winkler M.-K.H., Volcke E.I.P. (2015). Modelling simultaneous methane and ammonium removal in a one-stage aerobic granular sludge reactor. 9th IWA Symposium on Systems Analysis and Integrated Assessment (Watermatex 2015), 4-17 June 2015, Queensland, Australia

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Castro-Barros C.M., Daelman M.R.J., Mampaey K.E., van Loosdrecht M.C.M., Volcke E.I.P. (2013). Dynamics of N_2O emission from partial nitritation-anammox in a full-scale granular sludge reactor. 9th International Conference on Biofilm Reactors, IWA and Veolia Water, May 28-31 2013, Palais des Congrès d'Issy Les Moulineaux, Paris, France

Poster contributions presented by Celia Castro Barros

Castro-Barros C.M., Daelman M.R.J., Mampaey K.E., van Loosdrecht M.C.M., Volcke E.I.P. (2015). Towards sustainable wastewater treatment: minimizing greenhouse gas footprint though innovative nitrogen removal. Brussels Sustainable Development Summit 2015 (BSDS2015), 19-20 October 2015, Royal Academy of Belgium, Brussels, Belgium

Castro-Barros C.M., Rodríguez-Caballero A., Volcke E.I.P., Pijuan M. (2014). Effect of nitrite on the N_2O and NO production on the nitrification of low strength NH_4^+ wastewater. RENEW meeting, Resource Recovery from Wastewater, Ghent University, 10 September 2014, Ghent, Belgium (POSTER AWARD)

Castro-Barros C. M., Moralejo-Gárate H., Campos J.L., Kleerebezem R., van Loosdrecht M.C.M., Mosquera-Corral A. (2013). Crude glycerol as substrate for bioplastics production by bacterial enrichments. 10th IWA Leading Edge Technology Conference (LET2013), 2-6 June 2013, Bordeaux, France

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